A PATCH AND VOLTAGE CLAMP INVESTIGATION OF THE RESPONSE OF THE C1 NEURONE OF 'HELIX ASPERSA' TO 5-HYDROXYTRYPTAMINE

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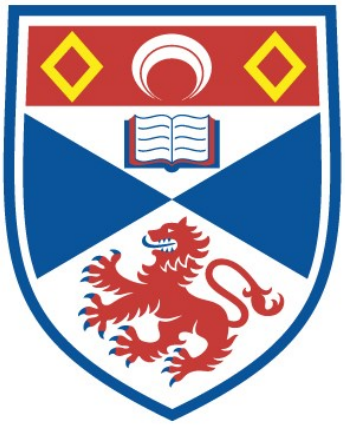

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A PATCH AND VOLTAGE CLAMP INVESTIGATION OF THE RESPONSE OF THE C1 NEURONE OF *HELIX ASPERSA* TO 5-HYDROXYTRYPTAMINE.

BY: MARGARET BARNES

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I Margaret Barnes hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

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I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 in October, 1983 and as a candidate for the degree of Ph.D. in October, 1983.

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ABSTRACT

Application of 5-hydroxytryptamine induces a voltage-dependent inward current in voltage clamped C1 neurones of Helix aspersa. This response has been shown to be the result of a decrease in K conductance and was studied using patch clamp and voltage clamp techniques.

Single channel K currents were recorded from cell-attached patches of the C1 neurone. Two sizes of unitary outward currents were commonly observed. The I-V relationships of both these unitary currents could be fitted by the Goldman-Hodgkin-Katz equation for a K current, having slope conductances of around 14pS and 54pS at +10mV, patch potential. Experiments, altering the K concentration in the patch pipette, or on the outer surface of isolated outside-out patches, suggested that these unitary currents were due to the flow of K^+ ions. Application of 5-hydroxytryptamine onto the C1 neurone, from outwith the patch pipette, reduced the activity of the larger K channels, recorded in the cell-attached patch. Both Ca-dependent, and Ca-independent K channels were observed on isolated inside-out membrane patches. It was unclear which of these types of channel corresponded to the 5-hydroxytryptamine sensitive channel in the cell-attached patch. Voltage clamp experiments also gave confusing results regarding the Ca-dependency of the 5-hydroxytryptamine response. However, in some C1 neurones 5-hydroxytryptamine caused a flattening of the "N" shaped I-V relationship, suggesting a decrease in the Ca-dependent outward current. The possibility that more than one type of K current was suppressed by 5-hydroxytryptamine was considered.

The effect of phosphodiesterase inhibitors was consistent with a mediation of the 5-hydroxytryptamine response by cyclic nucleotides. Injection of cAMP induced an inward current in the C1 neurone.

Single channel outward currents, which reversed at -50mV, were recorded from the A neurone. The activity of these channels was increased by 5-hydroxytryptamine, but their ionic nature was uncertain. Unitary outward currents of the M neurone were also recorded.

ABBREVIATIONS

ARC muscle	accessory radular closer muscle
4-AP	4-aminopyridine
cAMP	adenosine 3',5'-cyclic monophosphate
CNS	central nervous system
E_{Ca}	calcium equilibrium potential
E_{Cl}	chloride equilibrium potential
E_{K}	potassium equilibrium potential
E_{Na}	sodium equilibrium potential
EGTA	ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
epsp	excitatory post synaptic potential
GHK	Goldman-Hodgkin-Katz
gCa	calcium conductance
gCl	chloride conductance
gK	potassium conductance
gNa	sodium conductance
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IBMX	Isobutylmethylxanthine
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
I	current
I_{A}	A current
I_{C}	calcium-activated potassium current
I_{KD}	delayed rectifier potassium current
LSD25	lysergic acid diethylamide
MCC	metacerebral cell
5-MG	5-methoxygramine

7-MT	7-methyltryptamine
P_K	potassium permeability
(+)-TC	curare
TEA	tetraethylammonium
V	voltage
Vh	holding potential
Vc	command potential
Vm	membrane potential
Vp	patch potential

Units:

GΩ	gigaohm
pA	picoampere
pS	picosiemen
M	molar
mM	millimolar
ms	millisecond
mV	millivolt
µA	microampere
µM	micromolar
µm	micrometer
MΩ	megaohm
nA	nanoampere
s	second

SUMMARY

1. The voltage-dependent inward current response to 5-HT in the C1 neurone of Helix aspersa was investigated using both patch clamp and voltage clamp techniques.

2. Single channel outward currents were recorded from cell-attatched patches of the C1 neurone. Two sizes of outward channel currents were commonly observed. The smaller of these had a slope conductance of 14.2 ± 2.1pS at a patch potential of +10mV, whilst the larger had a slope conductance of 53.8 ± 3.3pS at a patch potential of +10mV.

3. The current-voltage relationships of single channel currents recorded from cell-attached patches of the C1 neurone could be fitted with theoretical relationships calculated from the Goldman-Hodgkin-Katz equation for K currents.

4. Experiments where the K concentration inside the patch pipette was altered suggested that the single channel outward currents were carried by K^+ ions.

5. Recordings of single channel currents made from isolated outside-out membrane patches, with solutions containing different K^+ concentrations, confirmed that the larger unitary outward currents were carried by K^+ ions.

6. 5-HT applied to the C1 neurone, from outwith the patch pipette, reduced the activity of channels, with a mean conductance of 54pS, in the cell-attached patch.

7. Perfusing the inner surface of isolated inside-out membrane patches with different free Ca^{2+} concentrations, revealed the presence of both Ca-dependent K channels and Ca-independent K channels. The Ca-dependent K channels had a slope conductance of 50 ± 11pS at a patch potential of +10 mV. The Ca-independent K channels had a slope conductance of 79 ± 15pS at a patch potential of +10mV.

8. Voltage clamp experiments were performed to investigate further the Ca-dependency of the 5-HT response. 5-HT reduced the calcium-dependent component of the outward current in some C1 neurones. This Ca-dependent component was observed as an "N" shape in the current-voltage relationship.

9. The effects of intracellular EGTA an Ca injection on the C1 neurone response to ionophoretically applied 5-HT were examined. The 5-HT response was not increased by Ca injection, but was decreased by EGTA injection at depolarised potentials.

10. 50μM verapamil had no effect on the response to ionophoresed 5-HT. However, the effect of a nominally zero Ca solution was inconclusive.

11. The possible role of cAMP as an intracellular messenger in mediating the 5-HT response was examined. Intracellular injection of cAMP produced an inward current similar to the 5-HT response. The phosphodiesterase inhibitors IBMX and theophylline also induced inward currents and blocked the response to 5-HT.

12. Single channel recordings were made from the A and M neurones and the effect of 5-HT application was observed. The A and M neurones receive synaptic input from the 5-HT containing C1 neurone.

13. Single channel currents, which reversed at around -50mV, patch potential, could be recorded from the A neurone. The activity of these channels was increased when 5-HT was microperfused onto the A neurone. The ionic nature of the channels recorded from the A neurone was not determined.

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CHAPTER 2 METHODS

CHAPTER 3 RESULTS

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 NEUROTRANSMISSION

In multicellular animals it is necessary to have a means of communicating between cells or groups of cells. In the nervous system, communication between neurones is usually rapid and may be of an electrical or chemical nature. The site at which interneuronal communication occurs is called the synapse. Electrical synapses involve the direct transfer, without measurable delay, of electrical information from one neurone to another. This usually takes place at sites seen, under electron microscopy, to resemble gap junctions.

At chemical synapses the transfer of information from one neurone to another typically has a delay of some milliseconds. It occurs by the release of a chemical substance from the terminals of the presynaptic neurone, which then alters membrane conductance at the postsynaptic site. Such substances are referred to as a transmitters or neurotransmitters. Transmitters were once thought to act by causing only increases in conductance to specific ions at the postsynaptic site. However, it is now apparent that neurotransmitters can also decrease ionic conductances at many sites. Peripherally, transmitters operate at the synapse between the neurone and effector organ.

The nature of the substances acting as neurotransmitters has been investigated. In 1964 Eccles reviewed a number of criteria which were thought to be important in establishing a substance as a neurotransmitter. These criteria included:

a) the substance, along with enzymes for its synthesis, should be present within the presynaptic neurone.

b) stimulation of the presynaptic neurones should result in the release of measurable quantities of the substance from the terminals.

c) the putative transmitter and the endogenous transmitter, released upon stimulation of the presynaptic neurones, must have the same effect on the postsynaptic neurones.

d) drugs which block the action of the transmitter candidate must also block the postsynaptic action of the endogenous transmitter.

e) finally, a mechanism should be present within the tissue for the termination of the action of the transmitter candidate.

In many preparations it is not possible to satisfy all of these criteria due to technical difficulties. For example the measurement of release may not be possible due to the small quantities involved. However, some compounds are generally established to have a neurotransmitter role. These include acetylcholine, noradrenaline, gamma-aminobutyric acid, 5-hydroxytryptamine (5-HT), glutamate and glycine. In addition,

the neurotransmitter role of many peptides is being established. Since this study concerns 5-HT, it is intended, in the following section, to review some of the evidence for the neurotransmitter role of 5-HT.

At some sites the effect of 5-HT and other neurotransmitters may be termed "modulatory". The term "modulation", as used in this thesis, refers to an action of a neurotransmitter which determines the effectiveness of other synaptic connections. The site of modulation can be at the presynaptic terminals, where the modulatory transmitter can effect transmitter release. It may also be at the postsynaptic site where it can alter the response of the cell to other inputs.

1.2 NEUROTRANSMITTER ROLE OF 5-HT IN MAMMALS

5-hydroxytryptamine (5-HT or serotonin) is an indolalkylamine which was first chemically characterised by Rapport in 1949 from extracts of blood serum. The presence of 5-HT was detected within the mammalian brain by Twarog and Page in 1953, using a bioassay on the clam heart (Venus mercenaria) and unidimensional paper chromatography. Amin, Crawford and Gaddum (1954) also established the presence of 5-HT within the mammalian brain by the use of bioassay and chromatography. The tissue which they used for the bioassay was more selective for 5-HT and they extended the study to determine the distribution of 5-HT within the central nervous system (CNS). The development of a histofluorescence technique by Falk, Hillarp, Thieme and Torp (1962) made it possible to directly

visualise 5-HT within neurones of the CNS. This technique involves the condensing of amines with formaldehyde, and, although it also causes primary catecholamines to fluoresce, 5-HT fluoresces with a longer wavelength, yellow colour enabling its distinction (Falk, 1962). Employing this technique, Dahlström and Fuxe (1965a,b) found that 5-HT was present in neurone somata, which were located largely in the brain stem raphe nuclei. Brain stem serotonergic (5-HT-containing) neurones project to most regions of the CNS (Fuxe, 1965). The findings of Dahlström and Fuxe (1965a,b) were confirmed and extended by Steinbusch (1981), using immunohistofluorescence. Serotonergic neurones, as well as being present in the brain stem raphe nuclei, were found in some areas outwith the brain stem (Steinbusch, 1981). Virtually all regions of the CNS from the frontal cortex to sacral spinal cord receive some innervation from serotonergic neurones, implying that 5-HT may have an important neurotransmitter function in the CNS.

Studies on the effect of serotonergic neurone stimulation and 5-HT application on postsynaptic neurones have provided further evidence for the neurotransmitter role of 5-HT in the mammalian CNS. Neurones of the rat hippocampus receive monosynaptic innervation from the median raphe nucleus, where large numbers of serotonergic neurones are located (Segal, 1975). Stimulation of the raphe neurones and ionophoresis of 5-HT inhibited the activity of hippocampal neurones in the CA1 region. Both of these actions were partially blocked by several 5-HT antagonists, including methysergide (Segal, 1975). Later studies on the hyperpolarising action of 5-HT on rat hippocampal neurones have indicated that it

is due to an increase in K conductance (gK) (Segal, 1980). Neurones of the amygdala and suprachiasmatic nucleus receive dense serotonergic innervation. Application of 5-HT onto the amygdala (Wang and Aghajanian, 1977) and suprachiasmatic nucleus (Bloom, Hoffer, Siggins, Barker and Nicoll, 1972), inhibited activity in these regions, mimicking the effect of raphe stimulation.

The role of 5-HT as a transmitter in the modulation of motor neurone activity has been studied. Studies of the effect of ionophoretic application of 5-HT onto motor neurones have often given confusing results, sometimes having no effect and sometimes causing inhibition or excitation (see review by Anderson and Proudfit, 1981). This confusion has arisen because the action of 5-HT on motor neurones is modulatory. 5-HT increases the excitability of the motor neurone to other inputs, but does not itself elicit action potential firing. McCall and Aghajanian (1979) studied motor neurone activity in the rat facial nucleus. They found that ionophoretic application of 5-HT alone failed to excite facial motor neurones. However, application of 5-HT dramatically facilitated subthreshold and threshold excitatory effects of ionophoretically applied glutamate on these cells. Additionally, 5-HT facilitated subthreshold and threshold excitation of facial motor neurones produced by stimulation of the motor cortex and red nucleus.

This facilitation by 5-HT was thought to be a specific receptor mediated effect since it was blocked by methysergide, which had no effect on noradrenaline produced facilitation. 5-HT released from terminals in the facial nucleus may be the physiological mediator of this effect since, p-chloroamphetamine, which causes the release of 5-HT also facilitated the excitatory effects of glutamate, but not after pretreatment with p-chlorophenylalanine, a 5-HT synthesis inhibitor (McCall and Aghajanian, 1979). The increased electrical excitability produced by 5-HT was associated with an increase in input resistance of the motor neurones (Van der Maelen and Aghajanian, 1980;1982). Experiments comparing the 5-HT response with responses to other transmitters, of known ionic mechanism, indicated that the most likely mechanism was a decrease in gK (Van der Maelen and Aghajanian, 1982). A similar modulatory effect of 5-HT has been described in rat lumbar spinal motor neurones (White and Neuman, 1980).

A neurotransmitter role for 5-HT has been established in the peripheral nervous system. In the coeliac ganglion of the guinea-pig, evidence suggests that the slow excitatory postsynaptic potential (epsp), produced by stimulation of the greater splanchnic nerves, is mediated by 5-HT in some cells (Dun and Ma, 1984; Dun, Kiraly and Ma, 1984). Application of 5-HT mimicked the effect of the slow epsp, even in low Ca high Mg solution, indicating that it was a direct postsynaptic effect. The 5-HT antagonist cyproheptadine blocked both the 5-HT response and the slow epsp.

Perfusion of the ganglion with 5-HT also blocked the slow epsp, presumably by affecting the same current. Fluoxeline, which blocks the reuptake of 5-HT and also L-tryptophan, a precursor of 5-HT, both enhanced the slow epsp, indicating that the synaptic response is due to the release of 5-HT. Furthermore, 5-HT immunohistofluorescence was detected in fibres surrounding many coeliac neurones (Dun, Kiraly and Ma, 1984). As with the 5-HT responses of the lumbar and facial motor neurones, those of the coeliac ganglion were associated with an increase in input resistance. The response was insensitive to intracellular Cl levels, and the most likely ionic mechanism was thought to be a decrease in gK (Dun and Ma, 1984).

In the myenteric plexus of the guinea-pig small intestine, the criteria of Eccles (1964) have been fulfilled for the establishment of 5-HT as a neurotransmitter (Wood and Mayer, 1979b). Wood and Mayer (1979a) described a slow epsp which occurred in tonic type myenteric ganglion cells upon electrical stimulation of interganglionic fibres. This slow epsp, which was accompanied by an increase in input resistance, resulted in the augmentation of excitability in myenteric neurones. Wood and Mayer (1979b) established that the slow epsp could be mimicked by application of exogenous 5-HT. The presence of 5-HT and tryptophan hydroxylase, the enzyme required for its synthesis, the release of 5-HT upon transmural stimulation and a high affinity uptake system for 5-HT in the myenteric plexus, have all been described previously by other workers, as discussed by Wood and Mayer (1979b). The studies of Wood and Mayer (1979b) completed the criteria of Eccles (1964)

for the establishment of 5-HT as a neurotransmitter in the myenteric plexus. Both the response to 5-HT and the slow epsp were blocked by the 5-HT antagonist methysergide, whilst tachyphalaxis, due to relatively high concentrations of 5-HT, also blocked the slow epsp, thus indicating that the slow epsp is due to a release of 5-HT from presynaptic neurones. The results discussed above therefore provide firm evidence for the role of 5-HT as a neurotransmitter in the regulation of gastrointestinal function.

1.2.1 MAMMALIAN 5-HT RECEPTORS

Although the different 5-HT receptor types are not of primary concern to this thesis, it is interesting to note that at least three types of mammalian 5-HT receptors have been described. 5-HT receptor heterogeneity was first established in the guinea-pig ileum by Gaddum and Picarelli (1957), who showed the existence of two receptor types which they named the 'M' and 'D' receptors. In later work two different 5-HT binding sites (5-HT_1 and 5-HT_2) were found in brain tissue (Peroutka and Snyder, 1979). The characteristics of the 5-HT_2 site have been found to correlate well with the 'D' receptor and also with the functional 5-HT receptor mediating a variety of responses in the brain. However, the 5-HT_1 site differs from both the 'M' and 'D' receptors and its functional significance is uncertain. Furthermore, it is becoming apparent that the 5-HT_1 binding site is not homogenous, and consists of at least three subtypes (Bradley, Engel, Fenuik, Fozard, Humphrey, Middlemiss, Mylecharane, Richardson and Saxena, 1986).

Table 1.1

Table summarising the classification of mammalian 5-HT receptors and their relationship to the "classical" receptors and the reported 5-HT binding sites. Some examples of neuronal responses thought to be mediated by the specific receptor types are given. The information in this table is summarised from Bradley et al, 1986.

RECEPTOR NOMENCLATURE	NEURONAL RESPONSES	SELECTIVE ANTAGONISTS	RELATIONSHIP TO "CLASSICAL" RECEPTORS	RELATIONSHIP TO BINDING SITES
"5-HT_1-like"	Autoreceptor-inhibition of 5-HT release (rat brain)			$5\text{-}HT_1$?
$5\text{-}HT_2$	Depolarisation of motor neurones (rat)	Ketanserin Cyproheptadine Methysergide	D	$5\text{-}HT_2$
$5\text{-}HT_3$	Stimulation of ACh release (guinea-pig ileum) Depolarization in superior cervical and nodose ganglion (rat and rabbit)	Cocaine MDL-7 2222 ICS 205-930	M	None

Three different 5-HT receptor types in the brain have been described from electrophysiological data (Aghajanian, 1981). The different nomenclatures have lead to confusion in the field of 5-HT receptors and a new classification (referring to receptors as '5-HT_1-like', 5-HT_2 and 5-HT_3) has been proposed by Bradley et al (1986). This classification and the functional role of the receptors in the mammalian nervous system are summarised in table 1.1. The 'M' receptor of Gaddum and Picarelli (1957) was renamed the 5-HT_3 receptor. The classification of the 5-HT_2 and 5-HT_3 receptors has been assisted by the finding of the selective antagonists ketanserin and MDL72222 (1α H,3α,5α H-tropan-3yl-3,5-dichlorobenzoate) respectively, for these receptors. However, due to a lack of selective antagonists, the receptors corresponding to the 5-HT_1 binding sites have not been well characterised and were therefore referred to as '5-HT_1-like' receptors (Bradley et al, 1986).

1.3 NEUROTRANSMITTER ROLE OF 5-HT IN INVERTEBRATE NERVOUS SYSTEMS

The presence of 5-HT in an invertebrate (molluscan) nervous system was first shown by Welsh (1954), using the clam heart as a bioassay. In a later study, with the more specific technique of spectrofluorescence, Welsh and Moorhead (1960) made a comparison of the 5-HT content of a number of invertebrate groups. Of the groups which they studied, the nervous systems of molluscs had the highest levels of 5-HT. Ganglia of bivalve molluscs had from 8 to 60μg/g tissue, while those of gastropod molluscs had 1.2 to 11μg/g

tissue. Annelid ganglia also had high levels of 5-HT (3.1 to 10.4μg/g tissue). However, the arthropods, echinoderms and protochordates had only small amounts of 5-HT (Welsh, 1968). As with the mammalian CNS, 5-HT within invertebrate nervous systems has been shown, by histofluorescence techniques, to be localised within neurones (Dahl, Falk, von Mecklenburg and Myhrberg, 1966; Rude, 1966; Sedden, Walker and Kerkut, 1968). In some preparations, for example molluscs, it is possible to locate identified serotonergic neurones (Cottrell and Osborne, 1970; Weinreich, McCaman, McCaman and Vaughn, 1973).

1.3.1 THE SUITABILITY OF THE MOLLUSCAN NERVOUS SYSTEM FOR TRANSMITTER STUDIES

The high levels of 5-HT present in the ganglia of molluscs (Welsh and Moorhead, 1960) suggests that 5-HT may have an important transmitter role in molluscs. The molluscan nervous system presents a useful preparation for the study of putative neurotransmitters. The neurone somata of molluscs are generally large and located on the outer surface of the ganglia. Neurones can often be identified by their size and position in the ganglia and the large size of many cells permits them to be isolated for biochemical studies (for example Cottrell and Powell, 1971). Furthermore, the ability to identify synaptic connections between specific neurones allows a comparison of the effect of stimulating an individual presynaptic neurone, with the effect of application of a putative neurotransmitter. For this reason, much work on the transmitter role of 5-HT has been carried out on molluscs, and one

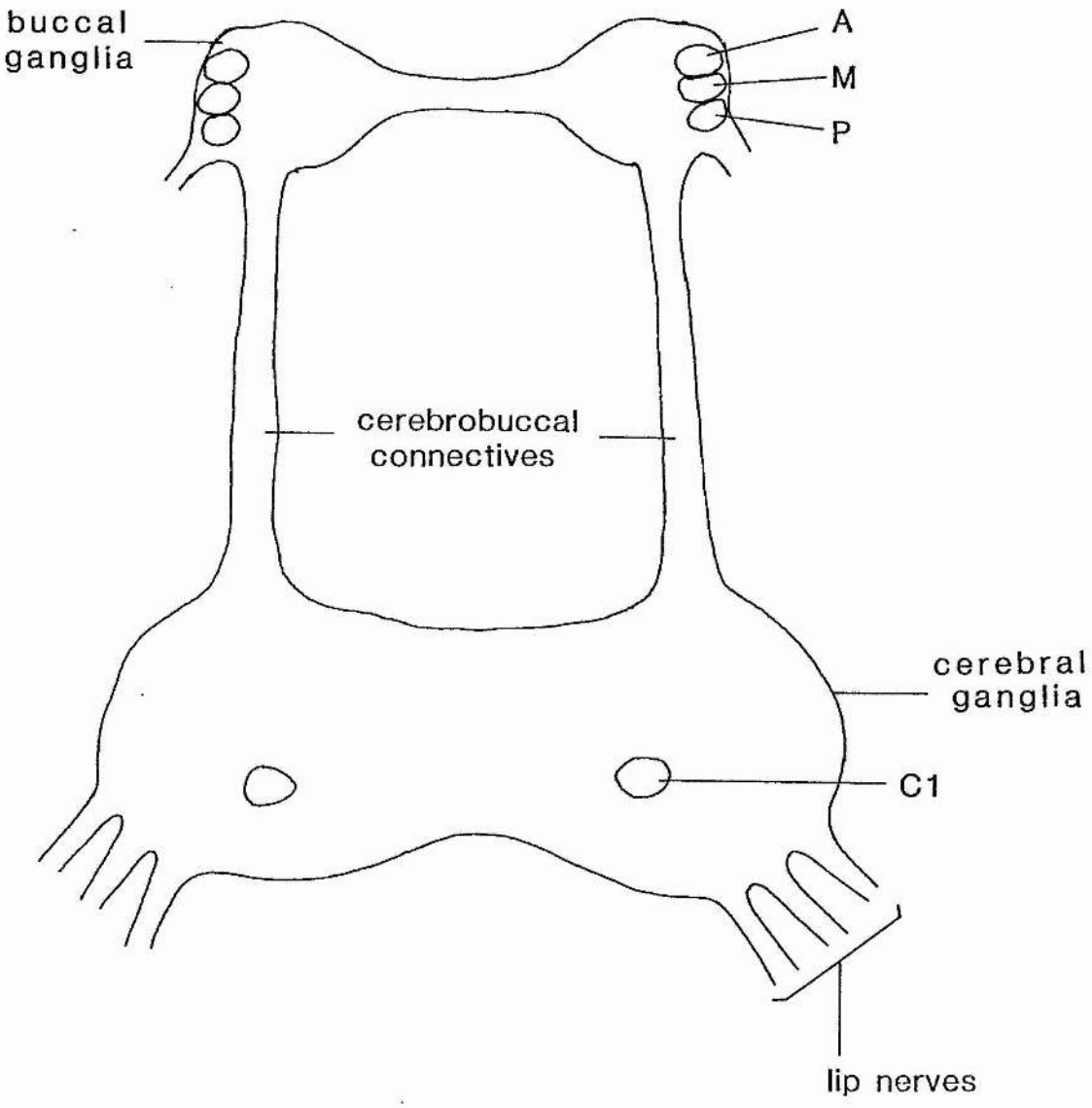
example of a specific synapse at which 5-HT has been shown to be the neurotransmitter is described below. Since the present study concerns a response to 5-HT in a molluscan neurone, much of the introduction which follows concentrates on molluscan systems, with references to relevant responses in other systems.

1.3.2. THE C1 NEURONE-BUCCAL NEURONE CONNECTIONS

In the pulmonate molluscs Helix and Limax, Cottrell and co-workers have carried out a detailed series of experiments on an identified serotonergic neurone and it synaptic connections (for reviews see Cottrell, 1977; Pentreath, Berry and Osborne, 1982). This large serotonergic neurone, located on the ventral surface of each cerebral ganglion, has been called the giant serotonin cell or neurone (GSC or GSN) and later, has been referred to as the C1 neurone. For the purpose of continuity, it shall be referred to as the C1 neurone here. The C1 neurone has been found to make synaptic connections with some neurones in the buccal ganglia (Cottrell, 1970; 1971; Cottrell and Macon, 1974). Since three of these follower neurones are large and easily identified by their position on the lateral edge of each buccal ganglion, they are suitable for making electrophysiological recordings of postsynaptic effects. Because of their positions, anterior, middle and posterior in the symmetrically located buccal ganglia (Cottrell, 1970), they are referred to as the A, M and P neurones (see figure 1.1).

Figure 1.1

Diagram showing the locations of the C1 neurones within the cerebral ganglia and the A, M and P (anterior, middle and posterior) neurones within the buccal ganglia. The C1 neurone could easily be identified by its size and position on the ventral surface of each cerebral ganglion. The A, M and P neurones could also be identified by their position on the lateral edge of each buccal ganglion.


buccal ganglia
A
M
P
cerebrobuccal connectives
cerebral ganglia
C1
lip nerves

The presence of 5-HT in the C1 neurone of Helix and Limax was detected using formaldehyde histofluorescence techniques (Cottrell and Osborne, 1970). Prior treatment with reserpine, which is known to deplete the 5-HT content of neurones, diminished the 5-HT fluorescence. Bioassays of extracts of individually dissected C1 neurone perikarya, established that each perikaryon in Limax and Helix contained about 1 ng of 5-HT (Cottrell and Osborne, 1970). Thin layer chromatographic analysis of ^{14}C-labelled dansylchloride treated homogenates of C1 neurones and P neurones indicated the presence of 5-HT within the C1 neurone but not in the buccal neurone (Osborne and Cottrell, 1971).

Biochemical studies on the C1 neurones of Helix pomatia have established the presence of the enzymes required for the synthesis of 5-HT. Isolated C1 neurones, incubated with tritiated 5-hydroxytryptophan, (5-HTP; the precursor of 5-HT) were able to convert this to 5-HT, but non-serotonergic neurones could not accumulate tritiated 5-HT when incubated with tritiated 5-HTP (Cottrell and Powell, 1971). Later it was shown that only 5-HT-containing neurones could specifically take up 5-HTP, whilst tryptophan can be taken up by all Helix neurones, but is only metabolised to 5-HTP in serotonergic neurones (Pentreath and Cottrell, 1973). Experiments on the homologous serotonergic cell in Aplysia (the metacerebral cell or MCC) have also established the presence of the enzymes required for 5-HT synthesis (Eisenstadt, Goldman, Kandel, Koike, Koester and Schwartz, 1973; Weinreich, McCaman, McCaman and Vaughn, 1973). It has been shown, from axonal

ligature experiments in both Helix and Aplysia, that 5-HT, synthesised in the soma can be transported down the axon towards the terminals (Osborne and Cottrell, 1970; Goldman and Schwartz, 1974). The release of 5-HT on stimulation of a single MCC has been detected in Aplysia by Gerschenfeld, Hamon and Paupardin-Tritsch (1976). Synaptic connections between the homologous serotonergic neurone and the buccal neurones have also been established in both Aplysia and Limax (Gerschenfeld and Paupardin-Tritsch, 1974b; Gelperin, 1975).

In view of the serotonergic nature of the C1 neurone, the synaptic connections which it makes with the A, M and P neurones of the buccal ganglia have been examined to determine if 5-HT is the transmitter substance. Stimulating the C1 neurone causes epsps in both the M and P neurones, whilst in the A neurone no clear epsps are seen but a series of membrane oscillations occur (Cottrell, 1971; Cottrell and Macon, 1974). The effects of C1 neurone stimulation can be mimicked by application of 5-HT onto the follower neurones. The interesting response of the A neurone was later shown to be a voltage-dependent depolarisation (Cottrell 1981). This shall be discussed in more depth in a following section concerned primarily with voltage-dependent responses. However, most work in determining the transmitter mediating the effects of C1 neurone stimulation has been performed on the synaptic connections between the C1 and M neurones, which results suggest to be monosynaptic (Cottrell and Macon, 1974).

Confirmation that 5-HT, which mimics the synaptic response, is the transmitter at this site was obtained from experiments using reserpine, which is known to deplete the 5-HT content of neurones. Injection of reserpine into animals over a period of 3 days, impaired, but did not abolish synaptic transmission between the C1 neurone and M neurone. The demonstration that antagonists which block the 5-HT response, also block the epsp was hampered by the lack of specific 5-HT antagonists. However, morphine was found to inhibit the response to both exogenous 5-HT and synaptic stimulation. The higher concentration of morphine which was required to block the epsp was thought to be due to a deeper location of postsynaptic receptors in the neuropile (Cottrell and Macon, 1974).

A mechanism for terminating the action of synaptically released 5-HT from the C1 neurone of Helix has been established. Autoradiographic studies revealed that terminal processes in the location of the synapses can selectively accumulate tritiated 5-HT (Pentreath and Cottrell, 1973). In Aplysia the uptake of 5-HT by the serotonergic MCC has also been shown (Gerschenfeld, Hamon and Paupardin-Tritsch, 1976). These results therefore establish a transmitter role for 5-HT in mediating the effects of the C1 neurone of Helix and the MCC of Aplysia. At these sites it has been possible to fulfil the criteria of Eccles (1964) for a single identified presynaptic neurone. Such a study, using identified pre- and postsynaptic neurones, has not been possible in the mammalian CNS.

1.3.3. MOLLUSCAN 5-HT RECEPTORS

As in mammals, the 5-HT receptors of molluscs have been found to be heterogeneous. In one study on neurones of Aplysia and Helix (Gerschenfeld and Paupardin-Tritsch, 1974a), six different responses to 5-HT were distinguished on the basis of ionic mechanism, time course and pharmacological properties. The different pharmacological properties suggested that at least four different receptors existed. Three of the 5-HT responses observed were depolarising and three were hyperpolarising, and, whilst four responses were due to a conventional increase in membrane conductance (an opening of ion channels), two others were due to a decrease in conductance (a closing of ion channels already open). A brief outline of these results is given below and summarised in table 1.2.

Two of the depolarising responses reversed at about 0mV, membrane potential (Vm) and were mediated by an increase in Na conductance (gNa). However, these two responses were distinguished by their time courses and pharmacological properties. The A response was a 'fast' depolarisation which was blocked by curare ((+)-TC), lysergic acid diethylamide (LSD25), tryptamine, 7-methyltryptamine (7-MT) and bufotenine. The A' response was a 'slow' depolarisation and of the above antagonists, only bufotenine was effective in producing its blockade. Two of the hyperpolarising responses were similarly mediated by an increase in conductance. The B response, a 'slow' hyperpolarisation, reversed

at -75mV and resulted from an increase in K conductance (gK). However, the 'fast' hyperpolarising response (C response) reversed at -56mV and was due to an increase in Cl conductance (gCl). The hyperpolarising responses could also be distinguished by their differing sensitivity to antagonists. The B response was blocked by LSD25, tryptamine, bufotenine and 5-methoxygramine (5-MG), whilst the C response was blocked by (+)-TC, LSD25, Tryptamine and neostigmine. A summary of these responses and their antagonists is given in table 1.2.

Two of the responses reported by Gerschenfeld and Paupardin-Tritsch (1974a) appeared to involve the closing of ion channels. The hyperpolarising β response, which reversed at about -30mV, was thought to be due to a decrease in both gNa and gK. The α response was a depolarising response which reversed at about -75mV and resulted from a decrease in gK alone. No antagonists were found for the α or β responses. However, these decreased conductance responses may also be specifically receptor mediated. In favour of this view is the fact that only certain identified neurones show these responses, and only when 5-HT is applied to restricted areas of these neurones. It is therefore unlikely that these responses represent an unspecific effect of 5-HT. The different pharmacological profiles of the four increased conductance responses (as summarised in table 1.2) indicate that these are mediated by four distinct receptor types.

Table 1.2

Table summarising the six different 5-HT responses of molluscan neurones, reported by Gerschenfeld and Paupardin-Tritsh (1974). The ionic mechanisms and the antagonists of these responses are shown.

RESPONSE	EFFECT	E_{rev}	IONIC MECHANISM	ANTAGONISTS
A	"Fast" depolarization	0	Increase gNa^+	(+)-TC, LSD25, Tryptamine, 7-MT, Bufotenin
A'	"Slow" depolarisation	0	Increase gNa^+	Bufotenine
B	"Slow" hyperpolarisation	-75	Increase gK^+	LSD25, Tryptamine Bufotenine, 5-MG
C	"Fast" hyperpolarisation	-56	Increase gCl^-	(+)-TC, LSD25, Tryptamine, Neostigmine
α	Depolarisation	-75	Decrease gK^+	
β	Hyperpolarisation	-30	Decrease gNa^+ and gK^+	

1.4 VOLTAGE DEPENDENT RESPONSES TO 5-HT

In molluscan neurones some responses to 5-HT show marked non-linearity in their current-voltage (I-V) relationship and are referred to as voltage-dependent responses. In LB and LC neurones of Aplysia, Pellmar and co-workers observed an unusual inward current response to 5-HT, which consisted of two components (Pellmar and Wilson, 1977; Pellmar and Carpenter, 1979). One component was a conventional Na-dependent inward current which decreased as the cell was held at more depolarised potentials, whilst the other component was a voltage-dependent inward current which only became apparent as the cell was held at depolarised potentials. An unusual response to synaptically released 5-HT has been observed in the A neurone of Helix on stimulation of the C1 neurone (Cottrell, 1971). This response has since been shown to have similar voltage-dependent properties to the response studied by Pellmar and co-workers (Cottrell, 1981;1982b). Other voltage-dependent 5-HT responses have been described in molluscan neurones (Klein and Kandel, 1978; 1980; Paupardin-Tritsch, Deterre and Gerschenfeld 1981; Deterre, Paupardin-Tritsch, Bockaert and Gerschenfeld, 1981;1982.) The 5-HT response in the C1 neurone of Helix, studied in this thesis, is an example of a voltage-dependent response (Cottrell, 1982b). In the following section these voltage-dependent 5-HT responses shall be discussed in more depth with particular regard to their ionic mechanisms.

1.4.1 THE 5-HT RESPONSE IN *APLYSIA* LB AND RB NEURONES

Some cells of the LB and RB group of *Aplysia* neurones were found to elicit only the voltage-dependent component of the two component inward current response described by Pellmar and Wilson (1977) and Pellmar and Carpenter (1979). In these cells the ionic mechanism of this voltage-dependent response has been studied in more depth. The response to 5-HT was not significantly affected by changes in Na and Cl concentration and it has been concluded that it is most likely to be due to an increase in a voltage-sensitive calcium conductance (gCa) (Pellmar and Carpenter, 1980; Pellmar, 1984). Although similar voltage-dependent 5-HT responses in *Helix* neurones have been shown to be the result of a decrease in gK, (Paupardin-Tritsch *et al*, 1981; Deterre *et al*, 1981; 1982; Barnes, Cottrell and Dunbar, in preparation) this was thought unlikely to be the mechanism responsible for this response, as no reversal of the response at hyperpolarised potentials was observed, and it was not sensitive to changes in extracellular K concentration (Pellmar and Carpenter, 1980). However, the ionophoretic response observed at depolarised potentials was blocked in the presence of sea water containing Ba, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) to block K currents. In contrast, no blocking effect of these agents was observed on the effect of perfused 5-HT on the I-V relationship obtained by stepping Vm from -60mV to depolarised potentials for a duration of 2s. It was suggested by Pellmar (1984) that the block of the ionophoretic response by Ba, TEA and 4-AP was due to a voltage-dependent block of the inward current

when the cell was held at a steady state depolarised level. Both the negative slope region of the current-voltage relationship, (present in Ba, TEA, 4-AP sea water, due to a pronounced inward current) and its enhancement by perfused 5-HT, were diminished at holding potentials more positive than -60mV. The response to 5-HT and the negative slope region were both abolished by Co, suggesting that they were due to a Ca current. However, although the most likely explanation was that 5-HT caused an increase in a Ca current, the involvement of K^+ ions was not entirely ruled out by Pellmar (1984). The possible involvement of a second messenger in this response has been examined and will be discussed in a later section.

1.4.2 5-HT RESPONSE IN *APLYSIA* SENSORY NEURONES

In *Aplysia* sensory neurones a voltage-dependent response to 5-HT has been studied, with regard to a possible role for 5-HT in mediating facilitation at this site (Brunelli, Castellucci and Kandel, 1976; Klein and Kandel, 1978; 1980; Klein, Camardo and Kandel, 1982). Although recent results cast doubt on the transmitter role of 5-HT in mediating the facilitation (Kistler, Hawkins, Koester, Steinbusch, Kandel and Schwartz, 1985), the response to 5-HT is still of relevance to this thesis. Here emphasis shall be placed on the ionic mechanism of the response, which appears to differ from the response studied in *Aplysia* RB and LB neurones by Pellmar and co-workers.

Klein and Kandel (1978) studied the effect of 5-HT application and synaptic stimulation (by activation of connectives from the head) on the action potential durations of sensory neurone perikarya. In the presence of TEA, to block some K currents, the action potential duration was prolonged and the plateau phase accentuated. Under these conditions, 5-HT further accentuated the plateau phase of the action potential. This effect was thought to be the result of an increase in gCa, since the plateau phase of the action potential can be blocked by Ca channel blocking agents (Klein and Kandel, 1978). However, later evidence suggested that the increase in gCa was secondary to a decrease in gK (Klein and Kandel, 1980; Klein, Camardo and Kandel, 1982). Voltage clamp experiments were performed on sensory neurone somata, whose axons were ligated to improve the space clamp. Application of 5-HT produced no change in inward currents elicited by stepping Vm from -45mV to +10mV in the presence of TEA, 4-AP and Ba. However, with similar steps in Vm, in the absence of K channel blocking agents, 5-HT caused a decrease in the outward current and an apparent enhancement of the inward current. Ca currents, recorded after blocking K currents with intracellular $Cs,^{+}$ were also unaffected by 5-HT and it was therefore concluded that 5-HT did not directly enhance the Ca current, but produced its effect by reducing a K current. Likewise, the synaptically evoked response was also found to be due to a decrease in gK (Klein and Kandel, 1980). However, the synaptically evoked decrease in gK may not be mediated by 5-HT (Kistler, et al, 1985). Recent experiments using the Ca sensitive dye (arsenazo III) have shown that, 5-HT increases the

intracellular Ca transients elicited by depolarising steps under voltage clamp conditions. Since the sensory neurone soma were voltage-clamped, this increase in intracellular Ca could not have been secondary to a decrease in gK. The mechanism by which intracellular Ca was increased was not established (Boyle, Klein, Smith and Kandel, 1984).

1.4.2.1 A NOVEL K CURRENT REDUCED BY 5-HT

The K current suppressed by 5-HT in Aplysia sensory neurones was reported to be a novel K current whose properties differed from the previously described K currents of molluscan neurones (Klein, Camardo and Kandel, 1982). The previously described K currents include, the fast transient K current (I_A), the delayed rectifier K current (I_{KD}) and the Ca-dependent K current (I_C) (for a review see Adams, Smith and Thompson, 1980). This novel 'serotonin sensitive' current was termed the S current (Klein, Camardo and Kandel, 1982). The S current could be distinguished from I_A on the basis of activation and inactivation kinetics. It was slower to activate than I_A and unlike I_A did not inactivate throughout a 300ms pulse. Similarly, the S current was distinguished from I_{KD} on the basis of kinetics, since I_{KD} decayed to a steady state level during an 800ms pulse, whereas the S current did not decay throughout an 800 ms pulse. Moreover, the S current was unaffected by 10mM TEA, which is reported to block I_{KD} (Thompson, 1977).

The S current, although diminished by Ca channel blocking agents such as Co, Cd or Ni and by perfusion with a zero Ca solution, was shown to differ from I_C. It was insensitive to Ba, which is known to block I_C (Hermann and Gorman, 1979; Adams and Gage, 1980). In addition, I_C produced by intracellular Ca injection was insensitive to 5-HT, indicating that the 5-HT sensitive current and I_C were different. The lack of effect of Ba on the S current also suggested that it is different from the muscarinic sensitive M current, described by Brown and Adams (1980), and shown to be blocked by Ba (Adams, Brown and Constanti (1982b). These findings lead Klein, Camardo and Kandel (1982) to propose that the 5-HT sensitive current was a novel K current.

1.4.2.2 SINGLE K CHANNELS MODULATED BY 5-HT

Single channel K currents, thought to correspond to the S current have been recorded from membrane patches of Aplysia sensory neurones using the patch clamp technique of Hamill, Marty, Neher, Sackmann, and Sigworth (1981). These channels were closed in an all-or-nothing fashion by application of 5-HT to the cell (Siegelbaum, Camardo and Kandel, 1982). Channels which remained open in the presence of 5-HT maintained the same kinetic properties. The properties of single K channel currents recorded were consistent with the properties of the S current. The I-V relationship of the channels showed rectification, in agreement with the Goldman-Hodgkin-Katz constant field current equation (GHK equation) for a K current (Goldman, 1943; Hodgkin and Katz, 1949).

However, the probability of channel opening was only slightly dependent on membrane potential. The channels did not inactivate with prolonged depolarisation and were insensitive to intracellular Ca concentration. It was proposed that these channels may contribute to a background K current (Siegelbaum *et al*, 1982). Since 5-HT closed these channels from outwith the patch pipette, the involvement of a second messenger was suggested, as shall be discussed later.

1.4.3 VOLTAGE-DEPENDENT 5-HT RESPONSES IN *HELIX* NEURONES

Voltage-dependent responses to 5-HT in neurones of the gastropod mollusc *Helix* have also been reported (Cottrell, 1971; 1981; 1982a,b; Paupardin- Tritsch *et al*, 1981; Deterre *et al*, 1981; 1982). The response studied by Cottrell (1971; 1981; 1982a,b) is physiologically important since it occurs at an identified synapse, where the presynaptic neurone (the C1 neurone) is known to be serotonergic (Cottrell, 1977). As discussed previously, the serotonergic C1 neurone in the cerebral ganglion of *Helix* makes synaptic connections with three identified neurones of the buccal ganglion, the A, M and P neurones. The synaptic response observed in the A neurone was first described by Cottrell (1971) to be either an unusual series of membrane oscillations, or a depolarisation accompanied by action potential firing. This response was delayed in onset and persisted for some time after cessation of C1 neurone activity. Subsequently, it was established that this was a slow, voltage-dependent depolarising response, which increased in amplitude as the cell was depolarised (Cottrell,

1981).

In a voltage clamped A neurone, C1 neurone stimulation, or ionophoretic application of 5-HT evoked an inward current with a voltage-dependency similar to the response studied by Pellmar and co-workers in Aplysia neurones (Pellmar and Wilson, 1977; Pellmar and Carpenter, 1979; 1980; Pellmar, 1984). The response was very small or absent at potentials more negative than -50mV, was never observed to reverse with hyperpolarisation and increased markedly with depolarisation. Na^+ and Cl^- ions were not involved in this response since replacing the NaCl content of the saline with sucrose had no effect on the response (Cottrell, 1981; 1982b). The Ca channel blocking agents Co and Cd (1-10mM) blocked the response to applied 5-HT, suggesting an involvement of Ca^{2+} ions (Cottrell, 1981). Such a response could therefore be the result of a decrease in a Ca-sensitive gK or an increase in a voltage-sensitive gCa.

It was found that application of 5-HT onto the C1 neurone itself produced a voltage-dependent inward current response with similar properties to the A neurone response (Cottrell, 1982b). Since the C1 neurone could be easily exposed and identified, it was a suitable cell in which to study further this response. In addition, the response of the C1 neurone may be physiologically important, as it has recently been found that the symmetrically located C1 neurones make synaptic connections with each other to produce a similar voltage-dependent response (Cottrell, unpublished observations).

Studies on the ionic mechanism of the 5-HT response in the C1 neurone have established that it is due to a decrease in a gK and probably not the result of an increase in gCa. Pharmacologically isolated Ca and Ba currents, elicited by stepping the membrane from a holding potential of -60mV were unaffected by 5-HT (Barnes, Cottrell and Dunbar, 1985; Cottrell, unpublished observation). Single channel Ca currents, in cell-attached membrane patches were also unaffected by application of 5-HT to the cell (Cottrell and Dunbar, 1985). Evidence for the involvement of K^+ ions has been established from ion substitution and channel blocking experiments. Increasing the extracellular K concentration caused a decrease in the size of the response, which was also found to be partially blocked by 50mM TEA and completely abolished by 7mM Ba (Barnes, Cottrell and Dunbar, in preparation). However, the Ca-dependency of this response has not been established and is one of the topics of concern in this study.

The 5-HT response studied by Gerschenfeld and co-workers in identified neurones of the viscero-abdominal ganglionic mass of Helix aspersa also appears to result from a decrease in gK and has many properties similar to the responses of the C1 and A neurones. However, unlike the responses of the C1 and A neurones, the 5-HT response of these neurones was observed to reverse at about -50mV (Paupardin-Tritsch et al, 1981; Deterre et al, 1981; 1982). Although this reversal potential differed from the K equilibrium potential E_K (shown to be -75mV in these neurones; Gerschenfeld and Paupardin-Tritsch, 1974a), the response was believed to be due to a

decrease in gK since it was insensitive to changes in Na or Cl concentrations, but was sensitive to changes in K concentration. However, the shift in the reversal potential for higher K concentrations was somewhat smaller than that predicted by the Nernst equation. Paupardin-Tritsch et al (1981) reported that the 5-HT response was rather insensitive to the extracellular application of K channel blocking agents such as Cs and TEA. However, the response was blocked by Ba (Paupardin-Tritsch, et al, 1981; Paupardin-Tritsch, Colombiaoni, Deterre and Gerschenfeld, 1985). Furthermore, intracellular TEA, Cs and Ba blocked the response to 5-HT (Paupardin-Tritsch et al, 1981). The voltage-dependent, 5-HT-induced, inward current was sensitive to Ca channel blocking agents and changes in extracellular Ca concentration. However, prolonged intracellular injection of the Ca chelating agent, EGTA (ethylene glycol bis-(β aminoethyl ether)N,N,N',N'-Tetraacetic acid) was without effect. The authors therefore concluded that the 5-HT response was mediated by a decrease in a K current, which was sensitive to extracellular Ca^{2+} ions, but was perhaps not the Ca-dependent K current, which is sensitive to intracellular Ca levels (Paupardin-Tritsch et al, 1981). Deterre et al (1982) proposed that this response was similar to, or analogous to the 5-HT-induced reduction of the specific K current (S current) described by Klein, Camardo and Kandel (1982) in sensory neurones of Aplysia, but different from the response studied by Pellmar and her co-workers in Aplysia RB and LB neurones (Pellmar and Wilson, 1977; Pellmar and Carpenter, 1979; 1980).

1.5 MODULATORY ACTIONS OF 5-HT

A modulatory transmitter response may be defined as one which has little effect by itself, but which determines the effectiveness of other synaptic responses. Modulation can occur at the presynaptic terminals of a neurone, to alter transmitter release and thereby alter synaptic effectiveness. Modulation may also occur at a postsynaptic site by altering the membrane characteristics of the postsynaptic cell and thus modifying the effectiveness of other synaptic inputs. Although the term modulatory imposes some artificial constraints on the action of a transmitter, in some of the responses mentioned previously the actions of 5-HT may best be described as modulatory. For example, 5-HT has been found to greatly enhance the effect of glutamate on rat facial motor neurones. However, 5-HT alone did not excite these neurones (McCall and Aghajanian, 1979). In many instances modulatory transmitter effects appear to operate on voltage-sensitive conductances and involve intracellular messengers (Kupfermann, 1979), as shall be discussed.

1.5.1 PRESYNAPTIC FACILITATION BY 5-HT

Shimahara and Tauc (1975; 1976) described a specific synapse in Aplysia, at which presynaptic facilitation occurred. Stimulation of the tentacular nerves caused a facilitation of the synaptic connection between a test interneurone (T-neurone) and a giant neurone in the pleural ganglion. Other synaptic inputs were

unaffected, suggesting that the facilitation occurred at a presynaptic site by altering transmitter release from the T-neurone. Ionophoretic application of 5-HT mimicked the facilitation and perfusion with the 5-HT antagonist, LSD25, or 5-HT itself, blocked both the heterosynaptic facilitation and the facilitatory effect of ionophoresed 5-HT. Thereby suggesting that 5-HT may be the transmitter mediating the presynaptic facilitation.

The particular neurone mediating facilitation has been identified (Shimahara and Tauc, 1976) and, although its serotonergic nature has not been confirmed, its action was also blocked by LSD25. The facilitatory effect of 5-HT could be mimicked by application of cAMP or dibutyryl cAMP, and it was suggested that 5-HT acted via cAMP to increase gCa in the presynaptic terminals, thus enhancing transmitter release (Shimahara and Tauc, 1977).

1.5.1.1 PRESYNAPTIC FACILITATION OF THE GILL-WITHDRAWAL REFLEX OF *APLYSIA*

5-HT, as well as being involved in the modulation of synaptic inputs at a cellular level, has also been implicated in the modulation of some behavioural responses. The modulatory role of 5-HT in the defensive reflex of gill-withdrawal in *Aplysia* has been studied by Kandel and co-workers (Castellucci and Kandel, 1976; Brunelli, Castellucci and Kandel, 1976). Although recent evidence has indicated that 5-HT may not be the transmitter involved in

mediating the facilitation, (Kistler, Hawkins, Koester, Steinbusch, Kandel and Schwartz, 1985) 5-HT is capable of mimicking the facilitation.

Stimulation of the siphon of Aplysia causes withdrawal of the gill by direct monosynaptic connections between the mechanoreceptor sensory neurones of the siphon and the gill motor neurones (Castellucci and Kandel, 1976). This reflex habituates with repeated stimulation due to an attenuation of the epsps recorded in the motor neurone. The decreased size of the epsps reflects a reduction in the amount of transmitter released from the presynaptic terminals. Habituation can be overcome and the response sensitized if the head of the animal is stimulated or if the connective from the head is stimulated electrically. Quantal analysis showed the facilitation to be due to an increased transmitter release from the presynaptic terminals rather than a postsynaptic effect (Castellucci and Kandel, 1976). It was concluded that neurones from the head end made synaptic contact with the presynaptic terminals of the sensory neurones to facilitate transmitter release. 5-HT, but not octopamine or dopamine mimicked the facilitatory effect and the 5-HT antagonist cinansern blocked facilitation (Brunelli, Castellucci and Kandel, 1976).

Some interneurones capable of mediating facilitation have been identified and these include the L29 cluster (Hawkins, Castellucci and Kandel, 1981a,b; Hawkins, 1981). These neurones were thought to be serotonergic on the basis of their morphological similarity

to the known serotonergic metacerebral giant cell (Bailey, Hawkins, Chen and Kandel, 1981). However, recent work has shown that these neurones do not have 5-HT immunoreactivity (Kistler et al, 1985). Serotonergic fibres were detected in close proximity to the sensory neurones and in the neuropile below the sensory neurones, and it remains possible that other, as yet unidentified, serotonergic interneurones also mediate facilitation. The facilitatory action of L29 neurones may be by another transmitter with similar effects to 5-HT (Kistler et al, 1985).

The action of synaptic facilitation and 5-HT in decreasing gK and increasing action potential duration (leading to an increased Ca influx and enhanced transmitter release) has only been demonstrated in the cell bodies of mechanoreceptor sensory neurones. In order for such a mechanism to account for presynaptic facilitation, it would have to occur at the presynaptic terminals.

1.5.1.2 THE TAIL-WITHDRAWAL REFLEX OF APLYSIA

The tail-withdrawal reflex of Aplysia represents another behavioural response in which 5-HT may act in a modulatory fashion by causing presynaptic facilitation (Walters, Byrne, Carew and Kandel, 1983b). Walters et al (1983a) established that electrical, chemical or mechanical stimulation of the tail produced withdrawal of the tail due to monosynaptic connections between tail sensory neurones and motor neurones. Stimulation of another site within the tail caused a sensitisation of the reflex by what was thought to be heterosynaptic facilitation. Although sensitisation was

accompanied by an increase in input resistance of the postsynaptic cell, this alone was considered insufficient to account for the sensitisation, which was concluded to to be a presynaptic effect (Walters et al, 1983b).

Application of 5-HT onto tail sensory neurones mimicked many of the effects of a sensitising tail shock. 5-HT and synaptic facilitation both caused an increase in action potential duration in sensory neurones. Application of 5-HT onto virtually isolated sensory neurone somata induced a slow depolarisation, accompanied by an increase in input resistance. This depolarisation was voltage-dependent and was probably due to a decrease in gK, similar to that observed in sensory neurones of the gill— withdrawal reflex (Walsh and Byrne, 1984b). In an elevated K solution (75mM), the response reversed at -37mV, which was considered to be close to the predicted E_K value of -29mV. However, further experiments were required to establish fully the ionic mechanism (Walsh and Byrne, 1984b).

The actions of 5-HT on tail sensory neurones were thought to be mediated by cAMP (Walsh and Byrne, 1984a,b). Ocorr and Byrne (1985) found that both 5-HT and small cardioactive peptide B (SCP_B) were capable of increasing cAMP levels in tail sensory neurones and that both these substances produced voltage-dependent depolarising responses. However, the 5-HT analogue tryptamine and another molluscan peptide FMRFamide neither caused an increase in cAMP levels nor a depolarising response. Both 5-HT and SCP_B were capable of eliciting these effects and the nature of the

physiological transmitter remains uncertain (Ocorr and Byrne, 1985).

1.5.1.3 PRESYNAPTIC FACILITATION AT THE CRAYFISH NEUROMUSCULAR JUNCTION

In both the gill-withdrawal and tail-withdrawal reflexes of Aplysia the proposed presynaptic actions of 5-HT have been studied by recording the effect of 5-HT in the cell bodies of sensory neurones and assuming that the same effect occurs in the terminals of these cells. At the crayfish neuromuscular junction it has been possible to make intracellular recordings from motor neurone axons close to the synaptic terminals (Dixon and Atwood, 1985). 5-HT has been shown to cause presynaptic facilitation at crustacean neuromuscular junctions (Glusman and Kravitz, 1982). By making recordings close to the terminals of the motor neurones innervating the crayfish opener muscle, Dixon and Atwood (1985) have found that the mechanism of facilitation by 5-HT is different from that described in sensory neurone somata of Aplysia. A 5 minute application of 10^{-6}M 5-HT produced an enhancement of excitatory junction potentials in the opener muscle, which lasted for about one hour after the application of 5-HT. Concomitant measurements from motor neurone terminal regions revealed a Na-dependent depolarisation of 5-6mV in amplitude and which decayed over one half hour. No increase in the size and duration of the action potentials (as found in Aplysia sensory neurones) was detected and in most cells a decrease in the membrane resistance was observed (Dixon and Atwood, 1985). Presynaptic facilitation by 5-HT

occurred even in the absence of Ca and it was thought that the 5-HT-induced Na influx could alter intraterminal Ca levels, possibly by altering Ca sequestering mechanisms.

1.5.2 MODULATORY ACTIONS OF 5-HT AT POSTSYNAPTIC SITES

In the examples of modulation discussed previously an action of 5-HT at a presynaptic site has been suggested. Modulatory actions of 5-HT at postsynaptic sites are also proposed and some of these shall be discussed in the following section.

1.5.2.1 MODULATION OF INKING BEHAVIOUR IN APLYSIA

In the inking behaviour of Aplysia, 5-HT is thought to play a modulatory role by acting postsynaptically to enhance the activity of the ink motor neurones (Walsh and Byrne, 1985). Byrne (1981) established that both the defensive behaviours of gill-withdrawal and inking shared at least some common sensory neurones and interneurones. Stimulation of the mantle region can elicit both responses. However, some interneurones (such as L31) are specific for only one circuit.

Stimulation of interneurone L31 or stimulation of the skin or pleuroabdominal connectives produces a slow epsp in the inking motor neurones which is accompanied by an increased excitability, thus enhancing other synaptic inputs. Application of 5-HT onto the neuropile region below ink motor neurone L14, mimicked the slow epsp (Walsh and Byrne, 1985), indicating a possible role for 5-HT

as the transmitter here. The response of L14 to ionophoretically applied 5-HT reversed at -80mV (close to E_K) and changes in the extracellular K concentration produced shifts in the reversal potential, which were in agreement with changes in E_K. The response to 5-HT, applied to the neuropile region, was therefore established to be due to a decrease in gK (Walsh and Byrne, 1985).

1.5.3 MODULATION OF FEEDING BY THE SEROTONERGIC METACEREBRAL CELLS OF MOLLUSCS

1.5.3.1 HOMOLOGY OF THE SEROTONERGIC METACEREBRAL CELLS THROUGHOUT THE MOLLUSCA

A cell corresponding to the C1 neurone of Helix is present in the cerebral ganglia of Aplysia. This cell is referred to as the metacerebral cell (MCC) and was found to contain 5-HT (Weinreich, McCaman, McCaman and Vaughn, 1973; Cottrell, 1974). Weiss and Kupfermann (1976) carried out a detailed study to determine whether these cells are homologous among the two genera of Aplysia and Helix. They concluded that they had very similar characteristics and were almost certainly homologous. Evidence suggests that these cells in other molluscan species are also homologous (Kupfermann and Weiss, 1981). Studies carried out to determine the physiological role of the MCCs in Aplysia and Limax indicated an involvement of these cells in the modulation of feeding behaviour.

1.5.3.2 MODULATION OF FEEDING PATTERN BY THE METACEREBRAL GIANT CELLS OF *LIMAX*

The role of the MCCs of *Limax* in feeding has been studied by Gelperin and co-workers and is reviewed by Gelperin (1981). The MCCs were found to make synaptic connections with some motor neurones in the buccal ganglia which were involved in the initiation and maintenance of feeding behaviour (Gelperin, 1975). In a quiescent preparation, activation of the MCCs alone was not sufficient to activate B7 (a motor neurone to the buccal musculature) or to initiate feeding. The MCC, although unable to activate B7, produced an epsp, which, if B7 was depolarised towards threshold, was sufficient to produce action potentials. Moreover, in a preparation in which B7 was active, stimulation of the MCC increased the activity of B7. Studies on the feeding motor program of an isolated lip-brain-buccal ganglia preparation also showed that, although the MCCs could not initiate the feeding motor program, they did enhance it once started.

1.5.3.3 MODULATION OF FEEDING BEHAVIOUR BY THE METACEREBRAL CELLS OF *APLYSIA*

The MCCs of *Aplysia* have been studied with regard to their role in modulating feeding behaviour (Weiss, Cohen and Kupfermann 1975; 1978; Weiss, Mandelbaum, Schonberg and Kupfermann, 1979; reviewed by Kupfermann and Weiss, 1981). As with *Limax*, the MCCs of *Aplysia* make synaptic connections with some motor neurones in

the buccal ganglia, and although activity of the MCCs does not initiate feeding, it does enhance the activity of motor neurones to the buccal mass. The interval between bursts in motor neurones B15 and B16 is reduced by activity of the MCC.

The MCCs, as well as sending processes to the buccal ganglia, also send processes to the buccal musculature, and in Aplysia a peripheral role in the modulation of feeding by 5-HT released from the MCCs has been shown (Weiss, Cohen and Kupfermann, 1978). Tension recordings made form the accessory radular closer muscle (ARC) have shown that activation of the buccal neurones causes contraction of this muscle and that contraction is dramatically enhanced by MCC stimulation. Although MCC activity increased the firing rate of the buccal motor neurones, neither this nor presynaptic facilitation were considered sufficient to account for the increased force of contraction. MCC activation was also able to enhance ARC muscle contraction in response to brief pulses of acetylcholine.

No potential or conductance change could be measured in the ARC muscle on stimulation of the MCC, and it was proposed by Kupfermann and Weiss (1981), that the MCC could exert a direct effect on excitation-contraction coupling in this muscle. This effect is probably mediated by 5-HT which has been shown to be present in the MCCs of Aplysia and is released upon stimulation (Weinreich et al, 1973; Gerschenfeld, Hamon and Paupardin-Tritsch, 1976). Exposure of the ARC muscle to 5-HT mimicked the effect of MCC activation and, both MCC activation, and 5-HT increased cAMP

levels in this muscle. Cyclic AMP analogues and phosphodiesterase inhibitors also enhanced contraction, thus indicating that the effect of MCC stimulation is mediated by a 5-HT induced increase in cAMP (Weiss, Mandelbaum, Schonberg and Kupfermann, 1979).

A parallel action of the molluscan neuropeptide SCP_B has been suggested for the enhancement of contraction of the ARC muscle. SCP_B was found to be present in the ARC muscle and the nerves which innervate it. Like 5-HT, SCP_B caused an enhancement of contraction via a postsynaptic mechanism, which was accompanied by an increase in cAMP levels in the muscle. However, 5-HT and SCP_B were thought to act on different receptors (Lloyd, Kupfermann and Weiss, 1984).

1.5.3.4 MODULATORY ROLE OF THE C1 NEURONE OF HELIX

As discussed previously, the C1 neurones of Helix make synaptic connections with certain identified neurones in the buccal ganglia. The effect of C1 neurone stimulation on the A neurone is to produce a slow, voltage-dependent depolarising response that is likely to be the result of a decrease in gK (Cottrell, 1971; 1981; 1982b). The physiological role of this slow voltage-dependent synaptic response has been considered (Cottrell, 1982a). Constant current depolarising pulses of about 4.5s duration applied to the A neurone elicited action potentials. However, the A neurone displayed accommodation, the number of action potentials per depolarising pulse decreasing with repetitive stimulation. It was found that stimulation of the C1 neurone caused an increase in the number of action potentials per depolarising pulse in the A

neurone, thus partially overcoming accommodation. Rapid activation of the C1 neurone caused a small (about 2mV) depolarisation of the A neurone, but this was insufficient to explain the facilitation, since depolarising the A neurone by this amount did not cause facilitation. During C1 neurone activation, the action potential duration of the A neurone increased (Cottrell, 1982a). The increase in action potential duration, if also present at the presynaptic terminals of the A neurone, could have a facilitatory effect in the release of transmitter. However, a more likely effect of C1 neurone activation is to enhance other inputs onto the A neurone, thus producing an increase in the transmission of action potentials similar to the effect seen in overcoming accommodation.

Synaptic connections between the symmetrically located C1 neurones have recently been established (Cottrell, unpublished observations). Activation of one C1 neurone produces a small inward current in the contralateral C1 neurone when it is held under voltage clamp conditions at a depolarised potential. This response is probably the result of a decrease in gK, as is the response to exogenously applied 5-HT. The small amplitude of the synaptically evoked response may indicate that the site of the synapses is some distance from the soma. The C1 neurones, like the A neurones, display accommodation when depolarising pulses are applied. The synaptic links between the C1 neurones may help to overcome accommodation.

1.6 MODULATORY ACTIONS OF 5-HT IN VERTEBRATE SYSTEMS

1.6.1 EFFECT OF 5-HT ON LAMPREY MOTOR NEURONES AND PREMOTOR INTERNEURONES

In the midline of the lamprey spinal cord there is a column of 5-HT-containing neurones, which form a dense plexus of 5-HT varicosities. Dendritic branches of segmental motor neurones and premotor interneurones are present in this plexus in close proximity to the 5-HT varicosities, suggesting that the 5-HT pathway may affect these neurones. This possibility was investigated by Van Dongen, Grillner and Hokfelt (1986). In most motor neurones and premotor interneurones studied, application of 5-HT had no effect on resting membrane potential. However, 5-HT did attenuate the late phase of the afterhyperpolarisation following an action potential, which is thought to be due to a Ca-dependent K current. Such a response to 5-HT, if present physiologically, could be important in modulating the discharge frequency of these neurones, as the late phase of the afterhyperpolarisation is a major determinant of discharge frequency (Van Dongen, et al, 1986).

1.6.2 MODULATORY ACTIONS OF 5-HT IN MAMMALIAN SYSTEMS

Modulatory roles for 5-HT in mammalian systems have also been suggested. Most of these have been described in an earlier section and shall only be mentioned briefly here, with regard to their neuromodulatory role. The modulatory effect of 5-HT on rat facial motor neurones, described by McCall and Aghajanian (1979), and Van der Maelen and Aghajanian (1980), was an increase in excitability, such that it dramatically facilitated the excitatory effects of ionophoretically applied glutamate or other excitatory substances. Likewise, 5-HT modulated the activity of lumbar spinal neurones by increasing the excitability to glutamate activation (White and Neuman, 1980). Dun et al (1984a,b) described a slow epsp in the coeliac ganglia of the guinea-pig which appeared to be mediated by 5-HT and which resulted in the facilitation of a cholinergic fast epsp.

The slow serotonergic epsp recorded in neurones of the myenteric plexus of the guinea-pig may also have a modulatory role (Wood and Mayer, 1979a,b). During the slow epsp there is an increase in input resistance, augmented excitability and a blockade of post-spike hyperpolarisation. The slow epsp is thought to effect two types of Ca-dependent K current, that associated with Ca influx during the action potential, and that present at resting potential and contributing to the high resting conductance, low excitability state of the neurones (Grafe, Mayer and Wood, 1980). The reduction of this latter Ca-dependent K current could be the

mechanism involved in long term synaptic augmentation of excitability observed in these neurones.

1.7 MODULATORY ACTIONS OF OTHER NEUROTRANSMITTERS

1.7.1 MUSCARINIC SUPPRESSION OF K CURRENTS

Stimulation of the cholinergic preganglionic fibres of bullfrog sympathetic ganglia causes, in addition to a conventional fast epsp, a slow epsp which was accompanied by an increased input resistance of the ganglionic cells (Weight and Votava, 1970). The slow epsp reversed at -88mV, near to the predicted E_K, and increased in amplitude as the cell was depolarised, suggesting that a decrease in gK was the ionic mechanism involved.

The slow epsp was found to be mediated by muscarinic receptors and was mimicked by application of muscarinic agonists. The nature of the K current suppressed by muscarinic agonists was studied by Brown and Adams (1980), who found it to be a novel voltage-sensitive K current which differed from the other major K currents previously described, namely I_A, I_{KD} and I_C (see Thompson, 1977 and Adams, Smith and Thompson, 1980). This muscarinic sensitive current was therefore termed the M current (Brown and Adams, 1980; Adams, Brown and Constanti, 1982a; Adams and Brown, 1982). The M current was activated when Vm was more depolarised than -60mV and did not inactivate with time. It was suggested that the presence of this current would have a potential clamping effect on the neurones (Adams, Brown and Constanti, 1982a).

Adams and Brown (1982) showed that synaptically evoked currents in voltage clamped sympathetic neurones were the result of an inhibition of the M current. The physiological role of M current suppression by acetylcholine may be to enable the neurone to sustain the repetitive firing of action potentials (Adams and Brown, 1982; Brown, Gähwiler, Marsh and Selyanko, 1986). The presence and modulation of M currents has also been established in other tissues, such as rat sympathetic neurones, myenteric neurones and hippocampal pyramidal cells (Constanti and Brown, 1981; Brown and Selyanko, 1985a,b; Halliwell and Adams, 1982; Brown Gahwiler, Marsh and Selyanko, 1986).

In some tissues muscarinic agonists have been found to decrease not only the M current, but also the Ca-dependent K current which contributes to the afterhyperpolarisation following an action potential (Pennefather, Lancaster, Adams and Nicoll, 1985; North and Tokimasa, 1983; Cole and Nicoll, 1983; Brown *et al*, 1986). North and Tokimasa (1983) reported that ACh, as well as decreasing the resting gK of guinea-pig myenteric neurones, also reduced the afterhyperpolarisation resulting from a Ca-dependent gK. In bullfrog sympathetic ganglion cells, two distinct types of Ca-dependent K currents were shown to exist. These were the fast component (I_C) and a slower component contributing to the afterhyperpolarisation (I_{AHP}). I_{AHP} was reduced by muscarinic agonists, but was less sensitive to these compounds than the M current (Pennefather, *et al*, 1985).

In hippocampal pyramidal cells muscarinic agonists decrease both a Ca-dependent K current and the M current (Cole and Nicoll, 1983; 1984a; Halliwell and Adams, 1982; Brown et al, 1986). Cole and Nicoll (1984a) found that stimulation of the afferent fibres in the stratum oriens resulted in a cholinergic, slow, voltage-dependent depolarisation in hippocampal pyramidal cells. Accompanying this slow epsp was an increase in input resistance and a facilitation of repetitive firing as a result of a decrease in the Ca-dependent K current underlying the afterhyperpolarisation. Whilst the block of the afterhyperpolarisation could be mimicked by ACh and the Ca channel blocking agent Cd, only ACh caused the voltage dependent depolarisation, which was thought to be due to a decrease in the M current (Brown et al, 1986). The presence of an M current which is susceptible to muscarinic agonists has been described in hippocampal neurones (Halliwell and Adams, 1982).

1.7.2 SYNAPTIC BLOCK OF A TRANSMITTER-INDUCED gK AND A Ca-ACTIVATED gK

Kehoe (1985a,b) reported that activation of certain presynaptic neurones caused a decrease in both cholinergically activated and Ca-activated K currents in a postsynaptic neurone in Aplysia. The slow epsp produced by activation of the presynaptic neurones was shown to be the result of a decrease in a cholinergically activated gK. It reversed near E_K and was dependent upon the extracellular K concentration in agreement with the Nernst equation. The cholinergic nature of the K current

reduced by synaptic stimulation was demonstrated with cholinergic agonists and antagonists. Furthermore, if the neurone mediating the slow epsp was fired shortly before a neurone mediating a cholinergic increase in gK, the cholinergic hyperpolarisation was markedly reduced (Kehoe, 1985a). The presynaptic neurones mediating the slow epsp also mediated a reduction in a depolarisation induced K current. This current was thought to be Ca-activated since it was eliminated in Ca free saline and after prolonged intracellular injection of EGTA (Kehoe, 1985b).

1.7.3 OTHER MODULATORY ACTIONS

Most of the modulatory actions described above involve a decrease in gK. However, modulatory transmitters also operate by other mechanisms. As mentioned previously the response to 5-HT studied by Pellmar (1984) in Aplysia neurones is believed to result from an increased gCa. Ca^{2+} ions are known to regulate many processes inside cells, such as the release of neurotransmitters or hormones. Direct transmitter activated modulation of voltage gated Ca channels at nerve terminals can be a means of presynaptic facilitation (Shimahara and Tauc, 1977), as can the indirect effect on Ca currents via a reduced gK (Klein and Kandel, 1978; 1980). Recent evidence suggests that in sensory neurones of Aplysia 5-HT may indeed cause an increase in intracellular Ca which cannot be accounted for by a secondary effect of the decreased gK (Boyle, et al, 1984). However, whether this increased intracellular Ca, as measured by the Ca-sensitive dye Arsenazo III, reflects an influx of Ca, a decreased uptake of Ca into intracellular stores or a

release from intracellular stores is not determined. That Ca channels of excitable membranes can be modulated by neurotransmitters is certain. Most of the evidence for this comes from cardiac muscle, where noradrenaline and adrenaline increase the probability of channel opening during depolarisation, (for a review of the modulation of Ca channels see Reuter, 1983). Recently it has been found that 5-HT can enhance the Ca current of some Helix neurones (Paupardin-Tritsch, Hammond and Gerschenfeld, 1986). This effect of 5-HT is thought to be mediated by cyclic GMP (cGMP) since it was mimicked by the intracellular injection of cGMP or activated cGMP-dependent protein kinase. In addition it was also mimicked by an inhibitor of cGMP-dependent phosphodiesterase (Paupardin-Tritsch, Hammond, Gerschenfeld, Nairn and Greengard, 1986).

1.8 MEDIATION OF TRANSMITTER ACTIONS BY INTRACELLULAR MESSENGERS

Modulatory actions of transmitters (such as those described above) often occur more slowly and persist for a longer duration than do direct excitatory or inhibitory responses. Such slow responses are commonly mediated by intracellular second messengers (Kupfermann, 1979). Direct transmitter-receptor mediated responses involve an interaction of the transmitter with a receptor, which is thought to be located on the protein complex, which incorporates the ion channel. Second messenger mediated responses involve an interaction of the transmitter with receptors, which are spacially separate from the ion channel, thus requiring the production of an intracellular messenger. Intracellular messengers include, cAMP,

cGMP, Ca, diacylglycerol and inositoltrisphosphate. Most of these substances (except inositoltrisphosphate which causes a release of Ca^{2+} ions from intracellular stores) are believed to activate specific protein kinases, which in turn phosphonylate certain proteins. Phosphorylation of proteins at or near the ion channel may bring about a change in the activity of the ion channel.

In this section some evidence for the role of intracellular messengers in the mediation of transmitter effects shall be discussed. However, this discussion shall not be exhaustive as many responses believed to involve intracellular messengers shall not be discussed, but only those of relevance to this thesis.

1.8.1 5-HT EFFECTS MEDIATED BY INTRACELLULAR SECOND MESSENGERS

In 1972 Cedar, Kandel and Schwartz measured an increase in cAMP production in isolated ganglia of Aplysia on stimulation of peripheral nerve connectives. Although they could not determine if the origin of the increased cAMP production was neuronal or glial, they were able to conclude that it was the result of synaptic stimulation and not merely an effect of impulse activity in the nerves. In addition, perfusion of the ganglia with 5-HT or dopamine caused an increase in cAMP production, whilst other transmitters tested, glutamate, noradrenaline, histamine and the acetylcholine agonist, carbachol, were ineffective. It was therefore proposed that a transmitter released at the synapses could enhance production of cAMP in Aplysia ganglia (Cedar and Schwartz, 1972). Since these findings of Cedar and Schwartz (1972)

many neurotransmitter effects have been proposed to involve intracellular messengers.

1.8.1.1 cAMP IN THE GILL-WITHDRAWAL REFLEX OF APLYSIA

Studies on the mechanism of sensitisation in the gill-withdrawal reflex of Aplysia suggest an involvement of cAMP. Application of dibutyryl cAMP or injection of cAMP into cell bodies of sensory neurones mimics the enhancement of synaptic transmission, induced by stimulating the connectives from the head, or by application of 5-HT (Brunelli, et al, 1976). The broadening of the action potential following application of 5-HT or connective stimulation was also enhanced by application of the phosphodiesterase inhibitors isobutyl methyl xanthine (IBMX) and Ro-20-1724 (Klein and Kandel, 1978).

The actions of cAMP are thought to be mediated by protein phosphorylation via a cAMP-dependent protein kinase. The role of protein phosphorylation in facilitating transmitter release from sensory neurones was studied by injecting the catalytic subunit of cAMP-dependent protein kinase into the cells. This caused a broadening of the action potential and a decreased input conductance (presumably by decreasing gK), thus enhancing transmitter release (Castellucci, Kandel, Schwartz, Wilson, Nairn and Greengard, 1980). The action of a substance which can inhibit the effect of cAMP is of more use in determining if cAMP is the intracellular mediator of a response. Injection of protein kinase inhibitor, which specifically inhibits cAMP-dependent protein

kinase, was found to block the effect of 5-HT on Aplysia sensory neurones (Castellucci, Nairn, Greengard, Schwartz and Kandel, 1982). 5-HT and cAMP both decreased the novel K current, the S current (Klein and Kandel, 1982).

The role of cAMP in the closure of K channels has been studied at the single channel level (Siegelbaum et al, 1982; Camardo, Shuster, Siegelbaum and Kandel, 1983; Shuster, Camardo, Siegelbaum and Kandel, 1985). Siegelbaum et al (1982) observed that K channels in a cell-attached membrane patch were closed by application of 5-HT to the cell. This effect of 5-HT from outwith the patch pipette further suggested the involvement of an intracellular messenger. Injection of cAMP into the cell also caused closure of these K channels (S channels). Later experiments (Shuster et al, 1985) revealed that the purified catalytic subunit of cAMP-dependent protein kinase caused closure of S channels when applied to the inside surface of an isolated inside-out membrane patch. Many, but not all of the characteristics of the closure of S channels by the catalytic subunit were the same as the closure of S channels by 5-HT applied to the cell (Shuster et al, 1985).

1.8.1.2 cAMP IN THE TAIL-WITHDRAWAL AND INKING RESPONSES OF APLYSIA

The decrease in gK in tail sensory neurones and ink motor neurones, like that in the sensory neurones of the gill-withdrawal reflex, may be mediated by cAMP. Two possible transmitters (5-HT and SCP_B), which cause a decrease in gK in tail sensory neurones, increased cAMP levels in these cells (Ocorr and Byrne, 1985).

Application of forskolin (a specific stimulator of adenylate cyclase; Seamon, Padgett and Daly, 1981), or cAMP analogues also decreased gK in these neurones, mimicking the effect of 5-HT or heterosynaptic facilitation (Walsh and Byrne, 1984a,b). In ink motor neurones, application of the 8-bromo derivatives of either cAMP or cGMP mimic the decrease in gK produced by 5-HT application to the neuropile region below these cells. However, only cAMP is thought to mediate the effect of 5-HT as the inward current to 5-HT was blocked during the 8-bromo cAMP-induced inward current, but not during the 8-bromo cGMP-induced inward current. The phosphodiesterase inhibitors, IBMX and Ro-20-1724, as well as forskolin also mimicked the 5-HT response. Furthermore, low doses of these compounds potentiated the response to 5-HT, indicating the response to be mediated by cAMP (Walsh and Byrne, 1985).

1.8.1.3 ROLE OF cAMP IN THE 5-HT INDUCED DECREASE gK IN HELIX

The involvement of cAMP in the slow inward current produced by 5-HT in certain snail neurones was studied by Deterre et al, (1981). Intracellular injection of cAMP, but not cGMP or 5'-AMP evoked a slow inward current similar to the 5-HT response. Low doses (1-20 μM) of the phosphodiesterase inhibitor IBMX increased both the amplitude and duration of the 5-HT response, whilst higher doses (up to 100 μM) caused an inward current and blocked any further inward current response to 5-HT. Theophylline and Ro-20-1724 produced similar effects, suggesting an involvement of cAMP in the response. Experiments on homogenates of pooled identified neurones established that 5-HT was capable of

stimulating adenylate cyclase activity by 50-100%. Measurements of adenylate cyclase activity have also been performed on single cells using a microassay technique. In cell F1, both 5-HT and dopamine cause a decrease gK and in this cell both these substances produced a stimulation of adenylate cyclase, while in a neurone which never showed an inward current response to either transmitter, no stimulation of adenylate cyclase activity was detected. Increasing the intracellular cAMP by injection of cAMP, or stimulation of adenylate cyclase with forskolin, produced an inward current in cell F1. Saturation of this inward current blocked the effects of 5-HT and dopamine (Deterre et al, 1982). These results indicate that in this cell both 5-HT and dopamine produce an inward current via cAMP.

The synaptically induced decreases in gK studied by Kehoe (1985a,b) may be mediated by 5-HT, although not such substantial evidence for this has been shown. Bath application of a permeant cAMP analogue p-chlorophenylthio-cAMP (CPT-cAMP) or phosphodiesterase inhibitors mimicked the synaptic block of the Ca-dependent K current and thus blocked any further effect of synaptic stimulation. However injection of cAMP into the soma did not affect this current which was thought to originate in the axon (Kehoe, 1985b). Injection of cAMP into the cell or application of CPT-cAMP did mimic the synaptic decrease of the cholinergic K current which is also thought to be mediated by cAMP. The synaptically-induced block of this K current, as well as the effect of cAMP injection were enhanced and prolonged by bath application of phosphodiesterase inhibitors.

1.8.1.4 cAMP MEDIATION OF A 5-HT INDUCED INCREASE gK IN APLYSIA

The examples of cAMP-mediated modulation described so far have mostly concerned decreases in K conductances. However, cAMP-mediated increases in K conductances have also been reported. Extensive studies have been made on a 5-HT-induced increase in a potassium current in the R15 neurone of Aplysia, which appears to be mediated by cAMP (Adams and Levitan, 1982; Benson and Levitan, 1983; Lemos and Levitan, 1984; Lemos, Novak-Hofer and Levitan, 1985). 5-HT was found to increase the anomalously rectifying K current in R15. This effect of 5-HT, which is present at more hyperpolarised potentials reverses at E_K in solutions of different K concentration (Benson and Levitan, 1983) and is blocked by the K channel blockers Ba, Rb and Cs.

In R15 application of cAMP analogues mimicked the effect of 5-HT on the current-voltage curve whilst phosphodiesterase inhibitors potentiated the effects of low concentrations of 5-HT (Drummond, Benson and Levitan, 1980). Adams and Levitan (1982) established that cAMP mediated protein phosphorylation was necessary for the 5-HT evoked response. To demonstrate this they used protein kinase inhibitor (PKI), a protein which binds with high affinity to the catalytic subunit of cAMP-dependent protein kinase, thus inhibiting its activity. Injection of PKI into R15 neurones selectively inhibited the response to 5-HT whilst not affecting the non-cAMP mediated dopamine response (Adams and Levitan, 1982). Experiments using a GDP analogue (GDPβS), which

selectively inhibits adenylate cyclase by binding to the GDP regulatory site also indicate an involvement of cAMP. Injection of micromolar quantities of this substance into R15 selectively inhibited the 5-HT response (Lemos and Levitan, 1984). Biochemical studies on the R15 neurone have identified two phosphoproteins which are inseparable from the 5-HT response (Lemos, Novak-Hofer and Levitan, 1985).

Cyclic-AMP mediated increases in gK have been reported in snail neurones (DePeyer, Cachelin, Levitan and Reuter, 1982; Ewald, Williams and Levitan, 1985). DePeyer et al (1982) found that internal perfusion of voltage clamped neurones of Helix roseneri, with the catalytic subunit of cAMP-dependent protein kinase caused an increase in a Ca-dependent outward current which contributed to the "N" shape of the current-voltage curve. No effect on the inward currents was observed and it was concluded that protein phosphorylation caused an increase in the Ca-dependent K current.

A single channel study also lead to the conclusion that protein phosphorylation could increase the opening of Ca-dependent K channels in Helix neurones (Ewald, Williams and Levitan, 1985). In these experiments, isolated inside-out membrane patches were perfused with the catalytic subunit, thus indicating (as with the experiments of Shuster et al, 1985, described earlier) that the site of phosphorylation must be at or very near the K channel itself. For a review on phosphorylation of ion channels see Levitan, 1985.

1.8.1.5 ROLE OF cAMP IN THE 5-HT INDUCED Ca CURRENT OF APLYSIA RB NEURONES

The 5-HT induced increase in a voltage-dependent Ca current studied by Pellmar and Carpenter (1979;1980) can also be mimicked by intracellular injection of cAMP (Pellmar, 1981a). However, Pellmar (1981b) found certain inconsistencies with the theory that cAMP was the physiological mediator of the 5-HT response in these neurones. Intracellular injection of guanylyl imidodiphosphate (GMP-NP) which is reported to activate adenylate cyclase in Aplysia (Treistman and Levitan, 1976), reduced the 5-HT response instead of mimicking or enhancing it as would be expected. Phosphodiesterase inhibitors mimicked the 5-HT effect by producing an inward current. However, they also antagonised the 5-HT response even at low concentrations, which themselves failed to produce an inward current. An antagonist of adenylate cyclase, dithiobisnitrobenzoic acid also failed to affect the 5-HT response. It was therefore concluded that, although intracellular injection of cAMP could increase the same Ca current as 5-HT, the response to 5-HT was not mediated by cAMP (Pellmar, 1981b).

1.8.1.6 5-HT INDUCED SECRETION IN INSECT SALIVARY GLANDS

Interesting results have been found through studies on the intracellular messengers mediating 5-HT-induced secretion from blowfly salivary glands. 5-HT causes an increased production of cAMP in these cells. However, the role of cAMP is not certain, but

it is thought to be involved in mobilising Ca from intracellular stores. 5-HT also mediates the hydrolysis of the membrane lipids, phosphatidyl inositols, yielding two intracellular messengers, namely inositolphosphates and diacylglycerol (Berridge, Dawson, Downes, Heslop and Irvine, 1983; Berridge, 1983; 1986; Berridge and Irvine, 1984). Neurotransmitter mediated hydrolysis of phosphatidyl inositols have also been shown in many other tissues by Berridge and co-workers. The water soluble component of this hydrolysis, inositoltrisphosphate, can diffuse through the cytoplasm to the endoplasmic reticulum, from where it releases stores of Ca. Inositol trisphosphate mediated Ca release from intracellular stores has been shown by work on permeabilised cells such as permeabilised insuloma cells (see review by Berridge, 1984). The lipid soluble component, diacylglycerol remains within the membrane, where, along with Ca, it activates protein kinase C. The role of protein kinase C in mediating secretion from salivary gland cells is not established, however the Ca mobilised from the endoplasmic reticulum by inositol trisphosphate will enhance secretion. Protein kinase C, activated by phorbol esters has been shown in other systems to modulate ionic currents (DeRiemer, Strong, Albert, Greengard and Kaczmarek, 1985; Baraban, Snyder and Alger, 1985).

1.8.2 MODULATION OF ACTIVITY OF APLYSIA BAG CELL NEURONES

The bag cell neurones of Aplysia exhibit pronounced modulation of their activity on brief stimulation of the pleuroabdominal connectives, or exposure to peptides from the reproductive tract (Kupfermann and Kandel, 1970; Heller, Kaczmarek, Hunkapiller, Hood and Strumwasser, 1980). This modulation in those otherwise quiescent cells, takes the form of an afterdischarge of repetitively fired action potentials lasting about 30 minutes. Evidence strongly suggests a role for cAMP in mediating this effect. The occurrence of afterdischarge is associated with an increase in the total cAMP levels of bag cell somata (Kaczmarek, Jennings and Strumwasser, 1978) and the phosphorylation of at least two proteins has been found to be enhanced following onset of afterdischarge (Jennings, Kaczmarek, Hewick, Dreyer and Strumwasser, 1982). Afterdischarge can be generated by the application of cAMP analogues to bag cells (Kaczmarek et al, 1978), and microinjection of the catalytic subunit of cAMP-dependent protein kinase has been found to enhance the rate of rise and height of the action potential (Kaczmarek, Jennings, Strumwasser, Nairn, Walter, Wilson and Greengard, 1980).

Analysis of the ionic currents modulated by cAMP in these cells has established that the inward currents are unaffected, while the delayed outward K currents and the fast transient K current, I_A, are depressed (Kaczmarek and Strumwasser, 1984). Strong (1984) studied the suppression of I_A induced by forskolin

and phosphodiesterase inhibitors and established that these substances increased the rate of inactivation of this current. This effect was sufficient to account for the repetitive firing during afterdischarge, whilst the modulation of the other K currents could account for the increased duration and size of the action potential. A modulation of I_A has also been reported in type B photoreceptor cells of the nudibranch Hermissenda (Alkon, Acosta-Urquidi, Olds, Kuzma and Neary, 1983).

In Aplysia bag cell neurones, modulation of the Ca current by a protein kinase has also been shown. Unlike cAMP-dependent protein kinase which was found to decrease K currents without affecting inward currents (Kaczmarek and Strumwasser, 1984), protein kinase C has been found to enhance the Ca current of bag cells without affecting the K currents (DeRiemer et al, 1985). This effect was elicited either by injection of protein kinase C or by stimulation of endogenous protein kinase C by application of a tumor promoting phorbol ester. However, the physiological importance of this effect is not known.

1.8.3 MODULATION OF THE Na CURRENT BY cAMP

The Na current of molluscan neurones is also susceptible to modulation by intracellular messengers (Aldenhoff, Hofmeier, Lux and Swandulla, 1983; Connor and Hockberger, 1983; Swandulla and Lux, 1984; Kehoe, 1986). Intracellular injection of cAMP caused an increase in the inward Na current which displayed unusual voltage-dependency. The cAMP-induced current either decreased, or

remained constant as the cell was hyperpolarised. In the Helix neurones studied by Aldenhoff et al (1983), it was later established that cAMP simultaneously induced a decrease in gK, thus accounting for the lack of conductance change observed (Swandulla and Lux, 1984). A synaptically activated cAMP-induced Na dependent inward current has been reported in Aplysia neurones (Kehoe, 1986).

1.8.4 INTRACELLULAR MEDIATION OF TRANSMITTER EFFECTS IN MAMMALIAN SYSTEMS

Mammalian nervous systems do not present as ideal preparations for the study of intracellular messenger mediated effects as do molluscan nervous systems. The criteria suggested by Bloom (1975) for the establishment of a second messenger role included the production of messenger in the postsynaptic cells. In Aplysia bag cell neurones, ink motor neurones and the F1 neurone of the snail such changes in cAMP levels have been established (Kaczmarek, Jennings and Strumwasser, 1978; Ocorr and Byrne, 1985; Deterre et al, 1981). In mammalian systems the detection of changes in intracellular messenger at the level of an identified neurone has not been possible, but levels in certain areas of the brain can be measured. Using the hippocampal slice preparation, it has been possible to demonstrate that noradrenaline causes a three to four fold increase in cAMP levels which is probably associated with neuronal tissue and not glia (Segal, Greenberger and Hofstein, 1981). Noradrenaline blocks accommodation in hippocampal pyramidal cells by reducing the late afterhyperpolarisation (Madison and

Nicoll, 1982). This effect of noradrenaline can be mimicked by the 8-bromo analogue of cAMP. Recently, further evidence for the role of cAMP in mediating this effect has been reported (Madison and Nicoll, 1986). Activation of endogenous cAMP, by the injection of a stable GTP analogue, or application of forskolin, caused a reduction of the afterhyperpolarisation and accommodation. Application of the phosphodiesterase inhibitors, IBMX and Ro-20-1724 also decreased the afterhyperpolarisation and potentiated the action of noradrenaline on the afterhyperpolarisation. Additionally, SQ22,536, a substance which has been shown to decrease cyclase activity in mammalian platelets, slightly increased the size of the afterhyperpolarisations and reduced the blocking effect of noradrenaline on the afterhyperpolarisation (Madison and Nicoll, 1986). These results indicate the probable mediation of the noradrenaline effect on hippocampal neurones by cAMP.

Second messenger mediation of the muscarinic action on hippocampal pyramidal neurones has been considered, but remains undetermined (Brown et al, 1986). A block of the Ca-dependent afterhyperpolarisation as discussed above can be produced by cAMP or cGMP (Madison and Nicoll, 1982; 1986; Cole and Nicoll, 1984b), thus mimicking the muscarinic effect as well as that of noradrenaline. However, protein kinase C has also been reported to block the Ca-dependent afterhyperpolarisation (Baraban, Snyder and Alger, 1985), as well as inactivating a Cl current (Madison, Malenka and Nicoll, 1986). The necessary experiments to determine the mechanism of this muscarinic effect remain to be done.

1.9 AIMS AND OBJECTIVES

Many of the modulatory actions of 5-HT described in this introduction have involved a decrease in a K current. Previous experiments have indicated that the 5-HT response in the C1 neurone of *Helix* also involves a decrease in gK. The aim of this study was to record single channel K currents from the C1 neurone, using the patch clamp technique of Hamill, *et al* (1981), and to establish the ionic nature of the channels. The effect of 5-HT on these channels was investigated using the cell-attached patch configuration, whilst the Ca-dependency of the channels was examined using isolated inside-out patches of membrane. The calcium-dependency of the response to 5-HT was studied at a whole cell level using voltage clamp techniques and some investigations into a possible role of cAMP in its mediation were made.

An attempt was made to study the effect of 5-HT on single channel currents recorded in cell-attached patches of the A neurone. The A neurone exhibits a voltage-dependent response to 5-HT, which appears similar to the response observed in the C1 neurone (Cottrell, 1982b). It was therefore of interest to determine if the same channels were present on the A neurone and C1 neurone, and if they were effected by 5-HT in the same manner.

CHAPTER 2

METHODS

METHODS

2.1 PREPARATION

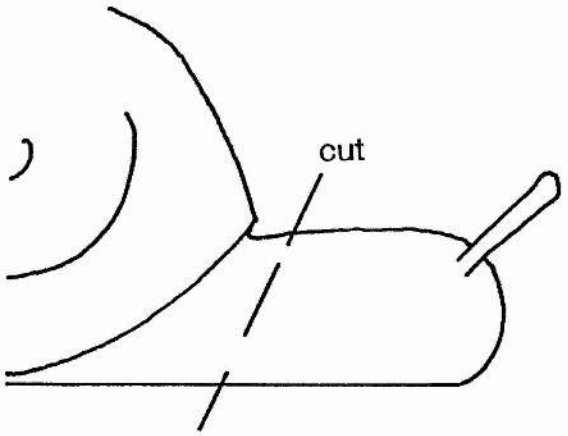
The experiments described in this thesis were performed on identified neurones in the ganglia of the common garden snail Helix aspersa. Specimens of H. aspersa were collected locally and kept in the laboratory until required. Animals which became inactive and hibernating during captivity were reactivated, by placing them in a moist environment before use in an experiment.

The identified neurones used in this study were either the C1 neurones of the cerebral ganglia or the A and M neurones of the buccal ganglia. Figure 1.1 shows diagrammatically the arrangement of the cerebral and buccal ganglia and the neurones within them. In most experiments, the cerebral ganglia alone were required. These were dissected from the animal by firstly cutting the head off as shown by the line in figure 2.1 and pinning it by the mouth to a wax dissecting dish. The oesophagus and buccal mass could then be identified and pulling the buccal mass with a pair of forceps revealed the circumoesophageal ring of ganglia. The location of the symmetrically placed cerebral ganglia above the oesophagus is shown in figure 2.1. The entire circumoesophageal complex of ganglia were dissected from the animal and transferred to the recording chamber for further dissection under a Nikon dissecting microscope. After removing the suboesophageal complex of ganglia, the cerebral ganglia were pinned, ventral side uppermost, to the Sylgard base of the recording chamber.

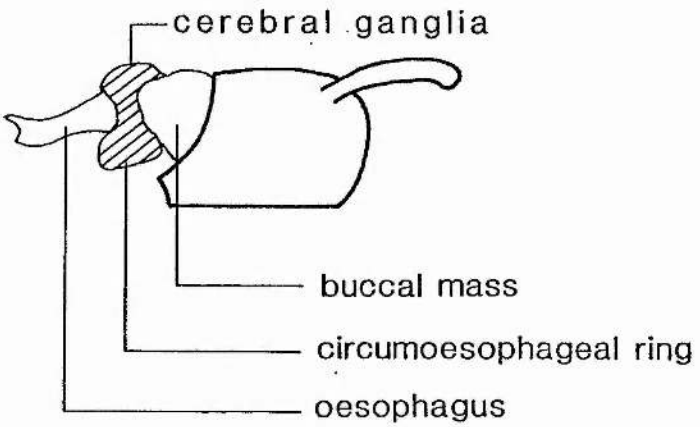
Figure 2.1

A, diagram showing the point at which the head of the snail was cut. This point was close to the shell, in an active snail which had its head stretched out. B, diagram illustrating the position of the circumoesophageal ring of ganglia, posterior to the buccal mass. The two symmetrically positioned cerebral ganglia lie on the dorsal surface of the oesophagus.

A


cut

B


cerebral ganglia
buccal mass
circumoesophageal ring
oesophagus

The ganglia of Helix are covered with two layers of connective tissue, of which the outermost layer is thicker and softer and could easily be peeled away using two pairs of forceps. This left the thinner, but tougher inner layer, through which the cells could be seen. For experiments using voltage clamp techniques, this layer was carefully ripped close to the C1 neurone, which could then to be impaled with microelectrodes. In patch clamp experiments a 0.1 to 0.2% trypsin solution (Sigma type 1X) was replaced for the normal solution before the final connective layer was ripped. The cells were then exposed to trypsin for 20-30 minutes, after which the trypsin solution was washed out by about 20ml of bath solution. Exposure of the cells to the enzyme trypsin made the surface more suitable for obtaining high resistance seals between the patch pipettes and cell membrane.

When buccal ganglia were used, the entire buccal mass was dissected from the animal along with the ganglia. The buccal ganglia are attached to the surface of the buccal mass and were carefully dissected free using fine scissors whilst viewing under the dissecting microsope. As much of the buccal mass as possible was removed to prevent movement of the preparation due to contraction of the buccal musculature. The remainder of the dissection was similar to that described for the C1 neurone.

2.2 RECORDING CHAMBER AND PERFUSION SYSTEM

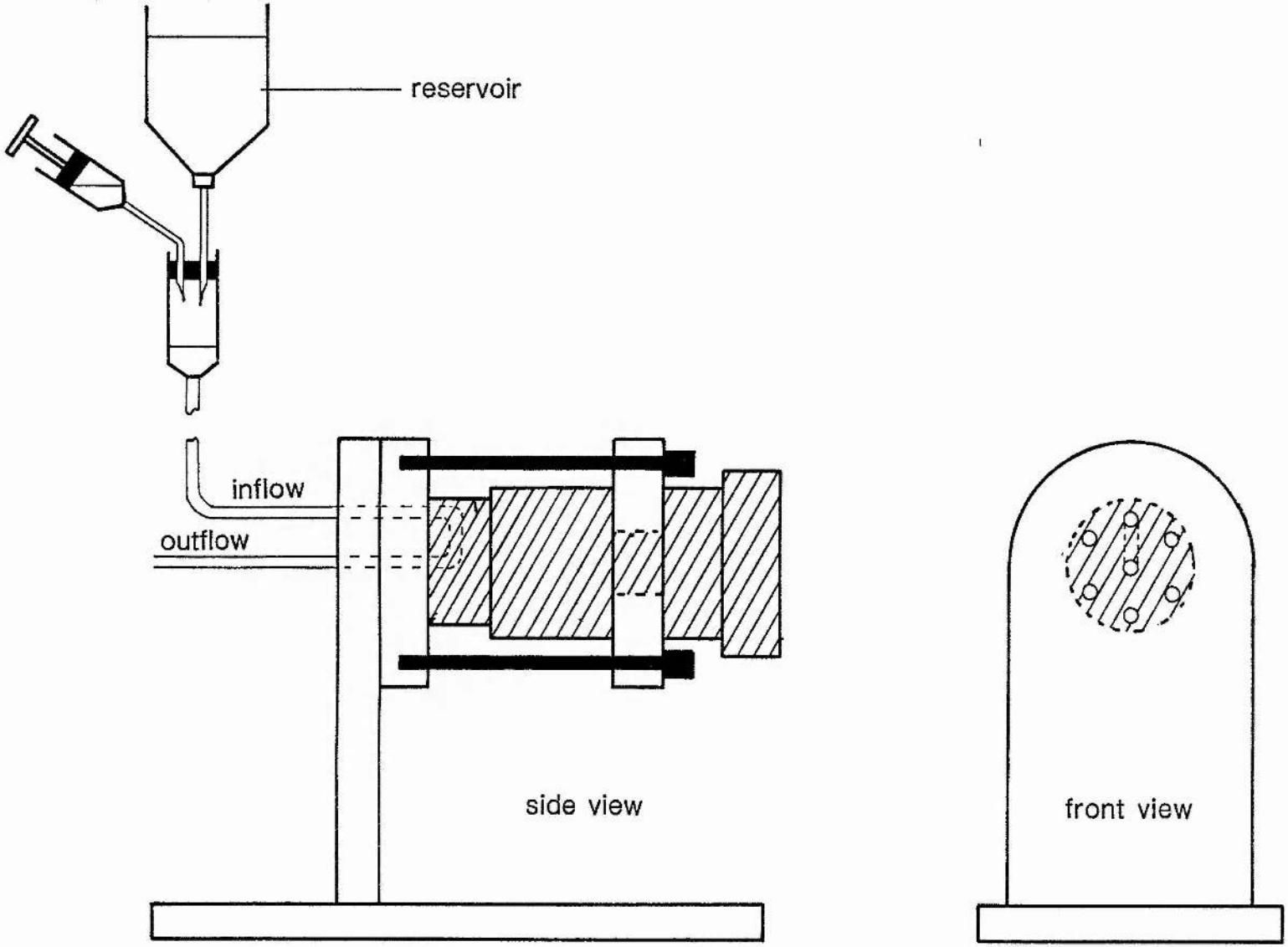
The recording chambers used were either a 1ml or 2ml chamber, cut out of Perspex and with a layer of Sylgard (Sylgard 184 silicone elastomer, Dow Corning), through which pins could be inserted. A diagram of the recording chamber is shown in figure 2.5. Illumination of the recording chamber was achieved by a tapered glass rod, connected at its thick end by a rubber bung to a dissecting light. The tapered end of the glass rod was positioned in the solution, close to the ganglia, to provide sufficient light to visualise clearly the neurones.

Inflow and outflow tubes to the recording chamber allowed the preparation to be perfused (or the isolated patch, in some patch clamp experiments). A six-way tap enabled the perfusing solutions to be changed during experiments. Six reservoirs (50ml syringes) of different solutions were connected, via smaller syringes and lengths of tubing, to the six-way tap. A diagram of the perfusion system, illustrating the mechanism of the six-way tap, is shown in figure 2.2. Rotating the movable portion of the tap allowed flow from a particular reservoir to be selected. The height of the reservoirs above the recording chamber (0.4m) provided the small force required for the flow of solution. The rate of flow could be regulated by adjusting a small clamp connected to the inflow tube to the bath. The inflow tube from the tap to the bath was kept as short as possible to minimise the time required for the exchange of solutions. The small syringes, interposed between the 50ml

Figure 2.2

Diagram of the six-way tap and reservoirs used to change the perfusing solution. Six reservoirs (50ml syringes) were connected to the inflows of the tap by lengths of plastic tubing. Only one reservoir is shown in this diagram. The inflows into the tap can be seen in the front view, surrounding the central outflow from the tap. The shaded portion of the tap can rotate and has a channel bored into it to connect the inflow and outflow. Rotation of this section of the tap allows the flow from a particular reservoir to be selected.

The height of the reservoirs above the bath created sufficient pressure for the flow of solution. The rate of flow from the tap to the bath was regulated by a clamp on the outflow tube from the tap.


reservoir
inflow
outflow
side view
front view

reservoirs and the tap made it possible to check that the solution was flowing freely during an experiment, by monitoring the drips.

Waste solution was sucked out of the chamber by the outflow tube, which was connected, via two aspirator flasks to an electrical pump. The outflow tube was positioned such that a small amount of solution always covered the ganglia. In some experiments, it was found to be better to have the outflow from the recording chamber flowing by capillary action, to a second small chamber, from which the waste could be sucked. Particularly in patch clamp experiments, flow out of the recording chamber by capillary action was beneficial, as it created less fluctuation in the fluid level and hence less movement. The normal bath solution used consisted of, 80mM NaCl, 5mM KCl, 7mM $CaCl_2$, 5mM $MgCl_2$, with 20mM Hepes buffer and made to pH 7.5 with NaOH. Solutions used in isolated patch experiments are listed in table 2.1. Other solutions used for patch clamp and voltage clamp experiments are referred to in the appropriate sections of the results.

2.3 PATCH CLAMP METHODS

2.3.1 PATCH PIPETTES

There are several stages in the fabrication of patch pipettes for obtaining high resistance, Gigaohm, seals with the cell membrane. These stages have been described by Hamill *et al* (1981) and in more depth by Corey and Stevens (1983). The method used here for making patch pipettes is described below.

Table 2.1

Table of solutions used for isolated patch experiments. The concentrations are given in mM. Solutions were adjusted to pH 7.5 by the addition of either NaOH or KOH. The particular alkali used is indicated in column 9. In experiments where the precise K^+ ion concentration was important, the volume of 1M KOH added was recorded in order to calculate the K concentration. The final K^+ and Ca^{2+} ion concentrations are given in columns 10 and 11.

SOLUTION	NaCl	KCl	$CaCl_2$	$MgCl_2$	SUCROSE	EGTA	HEPES	ALKALI	K^+	Ca^{2+}
1	80	5		5			20	NaOH	5	
2	3	82					20	KOH	96	
3	3	28			104		20	KOH	44	
4	3	73				5	20	KOH	100	6.7×10^{-10}
5	3	64	4			5	20	KOH	100	3.4×10^{-7}
6	3	62	4.7			5	20	KOH	100	1.3×10^{-6}
7	3	62	5			5	20	KOH	100	2.6×10^{-5}

2.3.1.1 PULLING PATCH PIPETTES

Initially, experiments were carried out using pipettes pulled from soda glass capillary tubes made for microhaematocrit purposes (Hawksley plain capillary tubes, cat. no. 1604). This soft glass has a lower melting point and is therefore easier to heat polish. However, the outer diameter of this tubing was rather inconsistent, resulting in pieces of tubing which could not be gripped in the chucks of the electrode puller, and which required varying heater settings to obtain the desired tip size. Borosilicate glass capillaries, supplied by Clark Electromedical (GC 150T-15) and designed for electrophysiological purposes, had a far more consistent diameter and produced more reliable patch pipettes. Although this hard glass requires higher temperatures to polish, it has better noise characteristics for electrical recording (Corey and Stevens, 1983).

Pipettes were pulled in two stages on a Campden moving coil microelectrode puller, model 753. An initial pull of about 5.3mm was made at a high coil temperature setting. This initial pull was achieved by placing a stop in the way of the moving clamp to prevent a complete pull. It resulted in a reduction in the thickness of the central portion of the capillary tube to about 0.2mm. The glass tube was then recentered in the heating coil and the second, complete pull made with a reduced heater setting. The delay before pull and force of pull were set to a minimum on the Campden electrode puller.

There was a considerable day to day variation in the temperature settings required to achieve the desired size of pipette tip. For this reason, it was found necessary to employ the "bubble test" described by Corey and Stevens (1983), on all pipettes pulled, to obtain an estimate of tip size. For this test, the pipette was connected to a 10ml syringe, via airtight polythene tubing, and the tip of the pipette placed in a small beaker of clean methanol. Pressure was applied to the pipette by depressing the plunger of the syringe until bubbles could be just seen emerging from the tip. The reading on the syringe at this point was taken as a qualitative indication of the tip size. Readings of 5-6ml on the syringe were considered suitable for patch pipettes.

In later experiments a Narishige PP83 puller, specifically designed for making patch pipettes, was used. Although this puller produced pipettes of a slightly longer taper, it was more consistent in pulling pipettes of the same tip size. No difference in the ability to obtain patches with either type of pipette was noticed.

2.3.1.2 COATING PIPETTES WITH SYLGARD

Covering the end of patch pipettes down to about 50μm from the tip, with a non-conducting resin, has been reported to reduce the noise characteristics of the pipettes (Hamill *et al*, 1981). Sylgard, silicone elastomer, was used for this purpose. A mixture of 10:1, resin to curing agent, by weight is recommended, but it

was found to be suitable to estimate these quantities. A small drop of Sylgard was spread around the area of the tip of the electrode, without touching the actual tip. The Sylgard coat was cured by moving the pipette up and down in the heating coil of a vertical electrode puller, on a low heater setting, for a few seconds.

2.3.1.3 POLISHING

In order to obtain stable Gigaohm seals, it is necessary to polish the tip of the pipettes. Gigaohm seals can be obtained with unpolished pipettes, but these are reported to be less stable (Hamill et al,1981). The process of polishing involves melting the tip of the electrode slightly to make the surface smoother, and burn off any Sylgard deposits, which have crept to the tip. Polishing was carried out by mounting the pipette on a microscope slide and viewing it under phase contrast at X250 magnification. The heat for polishing was supplied by a platinum-iridium wire, bent into a "U" shape and connected to a 6V, variable output power supply. A glass bead was melted onto the tip of the platinum-iridium wire to prevent metal deposits evaporating onto the pipette tip during polishing. The polishing apparatus was held in a manipulator, attached to an adjustable clamp, so that it could be positioned in view, under the microscope. A jet of air was directed to the point of the platinum-iridium wire to minimise fluctuations in the temperature of the wire due to drafts of air. The polishing apparatus is illustrated in figure 2.3, and an overhead view of the filament and pipette is shown in figure 2.4.

Figure 2.3

Diagram of the pipette polishing apparatus. The platinum-iridium filament was held by a manipulator on an adjustable clamp. This allowed the position of the filament to be adjusted to the level of the electrode. The electrode, placed on a slide on the microscope stage, could be finely positioned near to the filament. A 6V variable output power supply provided the power to heat the filament.

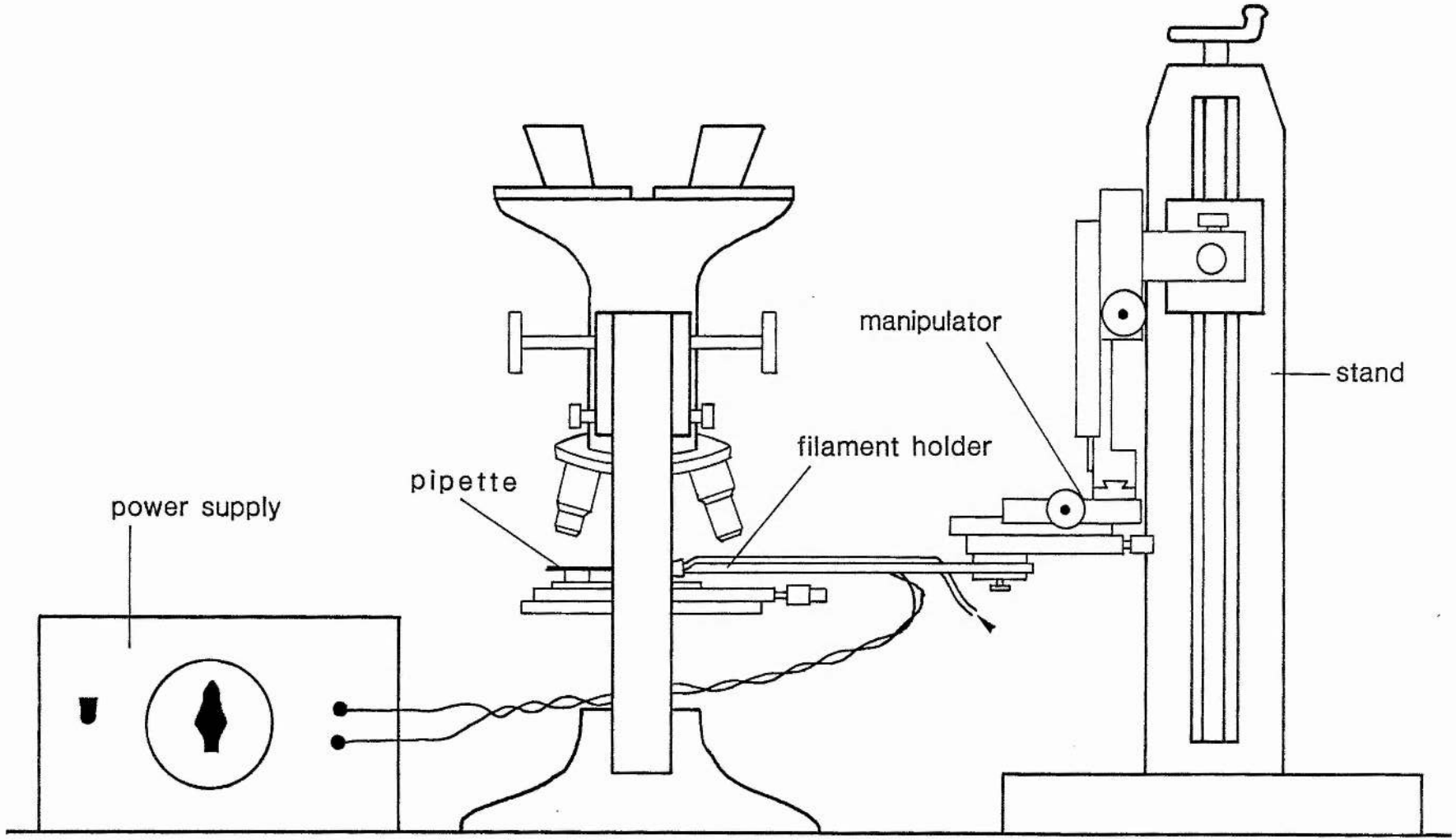
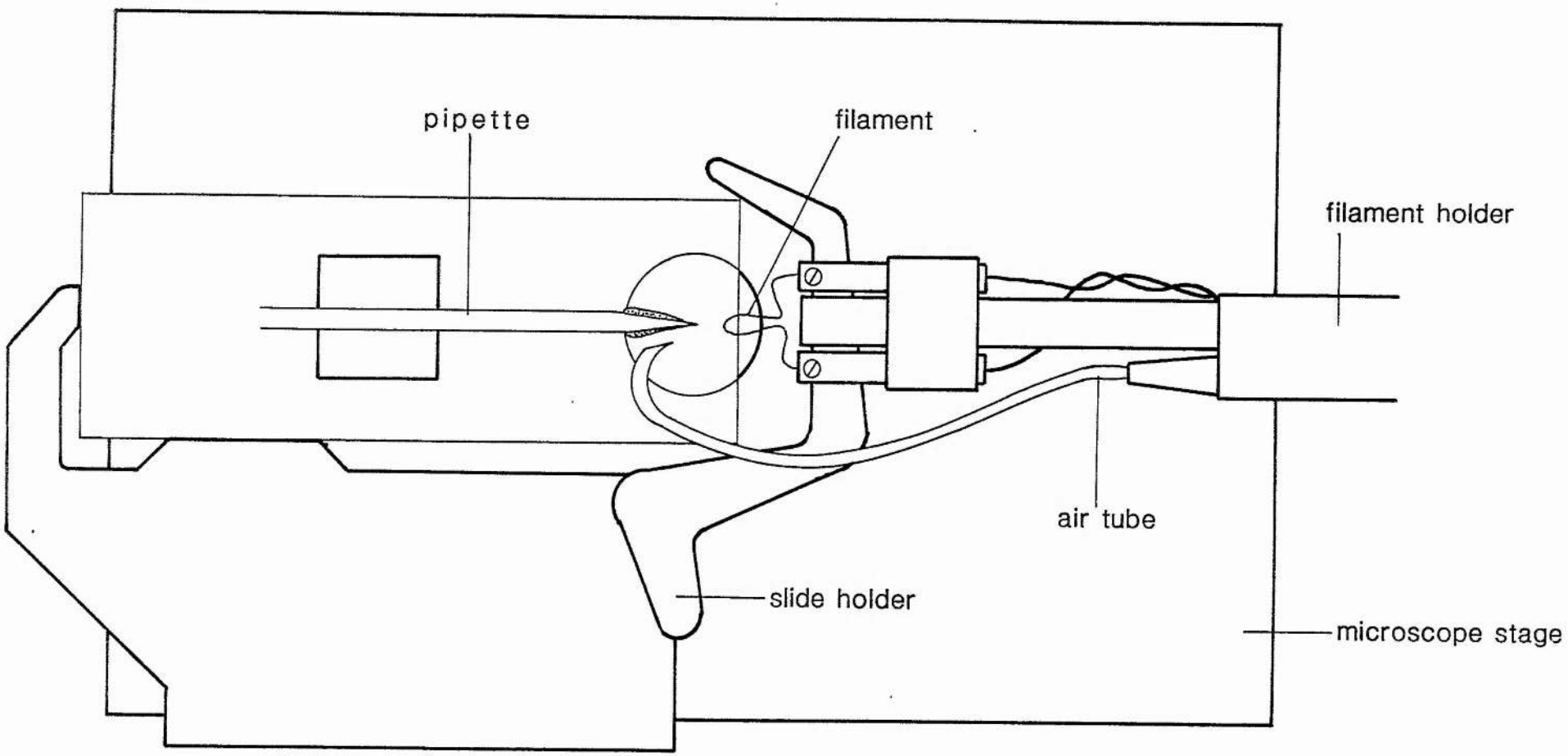

manipulator
stand
filament holder
pipette
power supply

Figure 2.4

Overhead view of polishing apparatus showing the position of the pipette on the microscope slide in relation to the platinum-iridium filament. The pipette could be positioned near to the heated filament by moving the microscope slide. The filament was bent into a U shape as shown and a jet of air was directed towards the tip.


pipette
filament
filament holder
air tube
slide holder
microscope stage

The pipette was kept some distance from the wire while it heated up, as it expanded on heating. The pipette was then moved close to the glowing wire until the tip could be seen to melt a little. After polishing a "bubble test" was performed, and pipettes of value 3-4ml on the syringe were considered sufficiently polished and good for patching. To keep pipettes clean, they were stored in Petri dishes until required, and were used within one or two days.

2.3.1.4 FILLING PIPETTES

Pipettes were always filled with filtered solutions to prevent pieces of debris blocking the pipettes or attaching to the tip and thus impairing the ability to obtain seals. Solutions were filtered with millipore filters of 0.22μm pore size (Millipore cat. no. GSWP02500). Pipettes were first filled by holding the tip in a small beaker of filtered saline and applying negative pressure to the pipette, using a syringe, until the tip was visibly filled. The remainder of the pipette was back-filled, using a syringe and hypodermic needle or thinly tapered polythene tubing. Bubbles caught in the tip were removed by flicking the pipette with the fore finger.

2.3.2 ELECTROPHYSIOLOGICAL METHODS AND APPARATUS

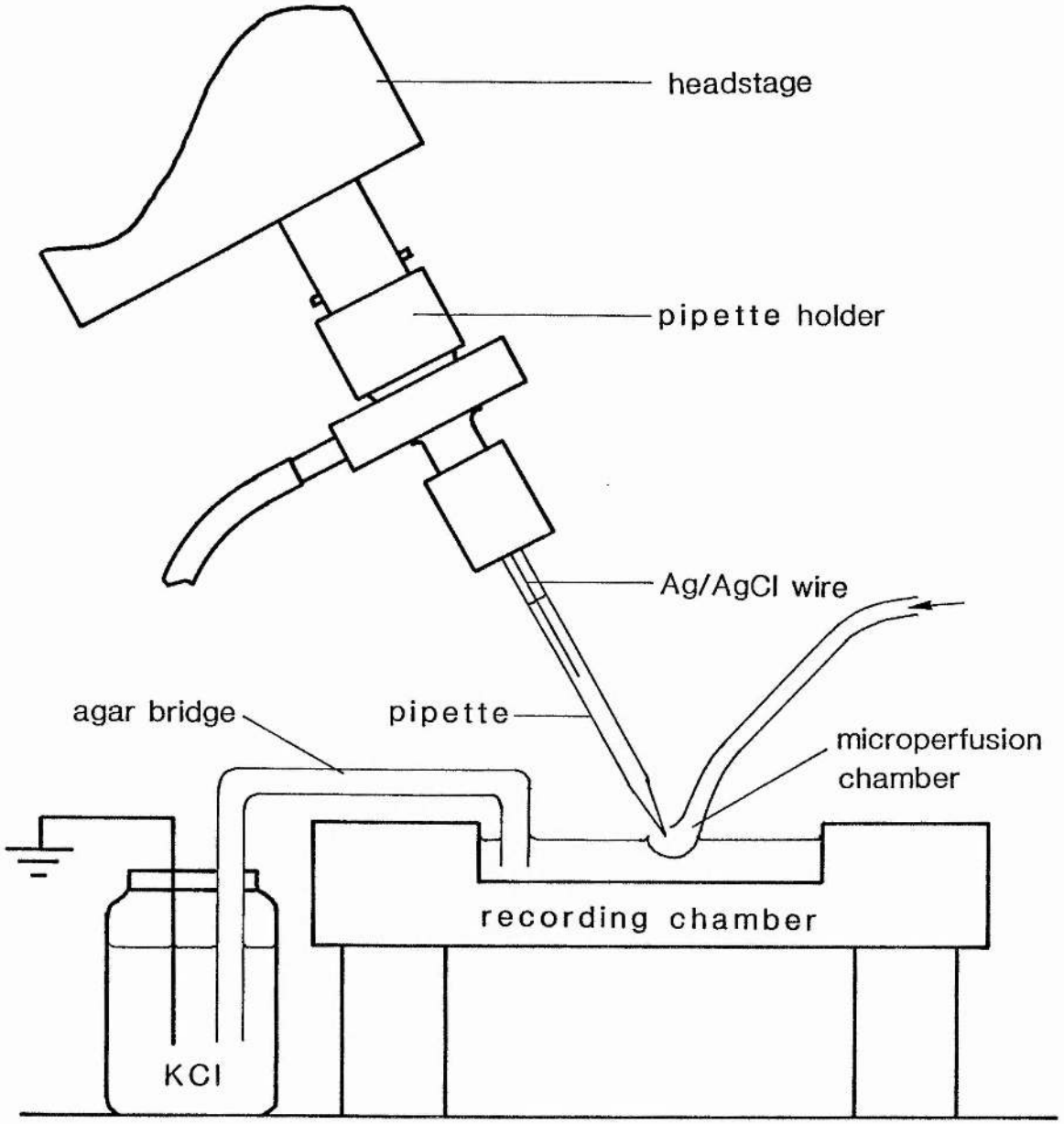
Patch clamp recordings were made using a List EPC5 patch clamp amplifier. During experiments, currents were monitored on a Gould (type 220) oscilloscope. A 1V pulse, supplied to the X10 stimulus input of the amplifier, from a Ceptu stimulator, could be used to measure electrode resistance and determine Gigaohm seal formation. Data was recorded on magnetic tape using a Racal 4DS FM tape recorder. In most experiments the filtering on the amplifier was set at 3KHz and data was recorded at 3.75 inches/s, wide band, on the tape recorder, giving a final filtering level of 2.5KHz.

The bath was grounded by an Ag/AgCl wire (made by dipping a Ag wire in molten AgCl), or an agar bridge, connected to the ground input of the amplifier headstage. The agar bridge was constructed from a glass tube, which was filled with a 3% agar solution in 1M KCl. One end of the agar tube was placed in the bath solution, while the other end was placed in a small container of 1M KCl, into which the Ag/AgCl wire was also placed (see figure 2.5). The ground input of the headstage is a high quality signal ground. In some experiments intracellular recordings of the membrane potential were made whilst recording from a cell-attached patch. These recordings were made using a DC Neurolog preamplifier N1 102. The electrodes for intracellular recordings were the same as used for voltage clamping (see later).

Figure 2.5

Diagram of the recording chamber with the agar bridge and microperfusion chamber. The agar bridge was used to connect the recording chamber to ground. The bridge consisted of a 3% agar solution in 1M KCl in a glass tube. This was placed with one end in the bathing solution and the other in a container of KCl, connected to the Ag/Agcl wire as illustrated.

The microperfusion chamber was used to perfuse isolated patches of membrane. The patch pipette was transferred into this small chamber, which was positioned just at the surface of the bath solution, to minimise contamination from the bath solution, but allow contact with the agar bridge.


headstage
pipette holder
Ag/AgCl wire
agar bridge
pipette
microperfusion
chamber
recording chamber
KCl

2.3.3 ARRANGEMENT OF EQUIPMENT

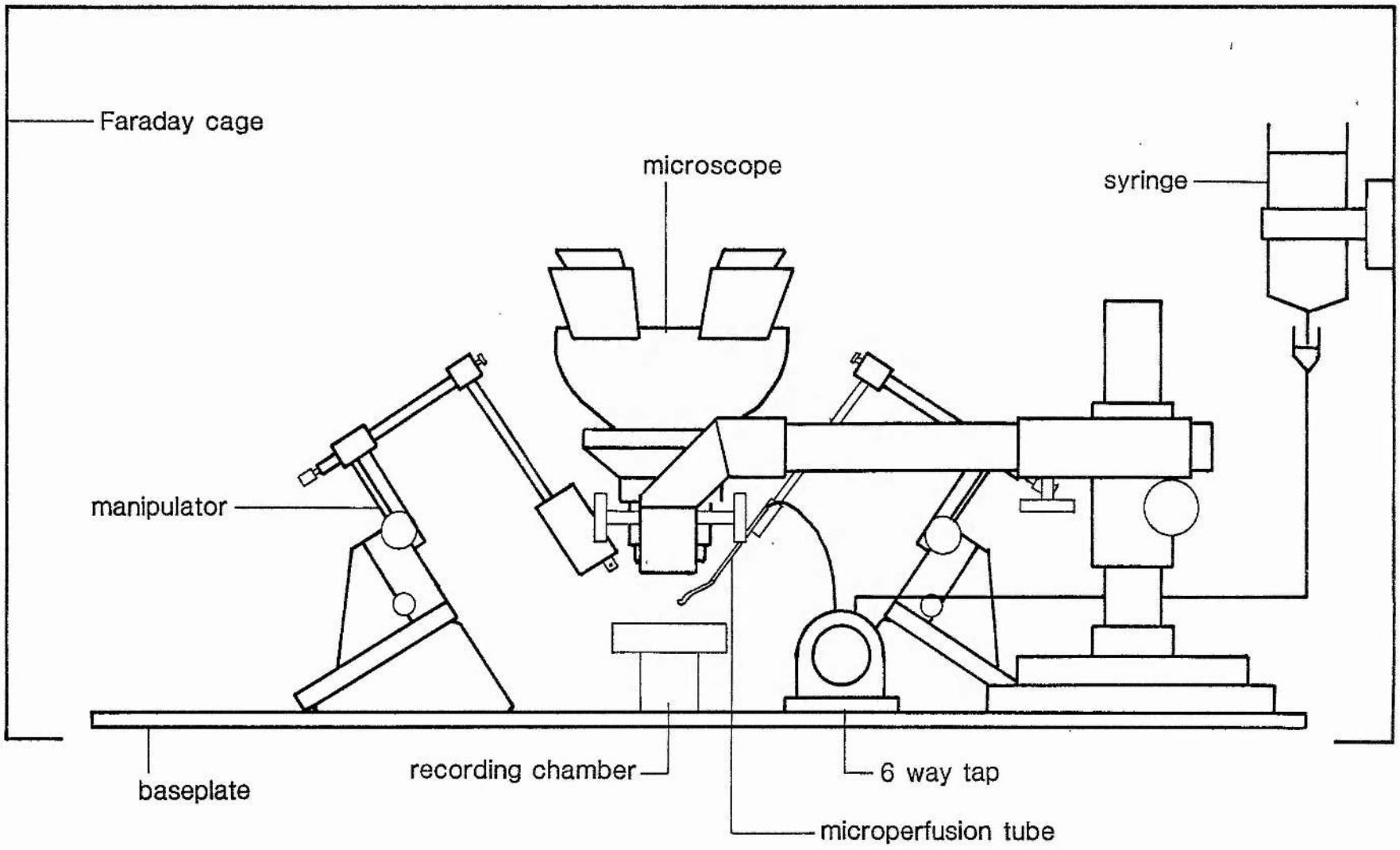
All of the equipment used around the recording chamber was set on a steel baseplate and enclosed in a copper Faraday cage. Pieces of equipment which could act as an aerial for electrical noise, such as the microscope and manipulators, were connected to a common ground, along with the baseplate and Faraday cage. The ground used was the high quality signal ground of the patch amplifier. This shielded the headstage of the amplifier, preventing the pick-up of electrical noise. During recording, further shielding from noise was obtained by bringing the front of the Faraday cage down. The arrangement of the equipment on the baseplate is shown in figure 2.6. The baseplate rested on a vibration isolation table (Wentworth laboratories). High pressure air from an air cylinder was used to fill the four pockets at each corner of the table, and so float the baseplate on cushions of air. The table was filled to a pressure of 1.4bar. The floating of the baseplate on cushions of air minimised vibrations transmitted through the floor. The electronic equipment was mounted on a rack to the side of the cage.

2.3.4 FORMATION OF A CELL-ATTACHED PATCH

Filled patch pipettes were placed on the Ag/AgCl wire of the amplifier headstage, and held by an airtight pipette holder, through which, positive and negative pressure could be applied by mouth, via a length of tubing. With the pipette in the bath

Figure 2.6

The arrangement of the equipment on the baseplate. The equipment was arranged on a steel baseplate and enclosed in a copper Faraday cage, both of which were connected to ground to sheild from noise. The Faraday cage had a front which could be brought down during recording, to further sheild from electrical noise. This is not shown. The baseplate rested on an air table (also not shown).


Faraday cage
microscope
syringe
manipulator
baseplate
recording chamber
6 way tap
microperfusion tube

solution, the resistance could be measured by passing a 1V pulse from the stimulator into the X10 stimulus input of the amplifier, and measuring the resultant current deflection. Pipette resistances were about 2 to 6MΩ. Pipettes with a resistance much higher than this were probably blocked and were discarded.

To form cell-attached patches, the pipette was carefully advanced down to the surface of the cell, using a Prior micromanipulator, while observing the current pulse on the oscilloscope. When the pipette touched the cell the size of the current pulse reduced. If gentle suction was applied at this point, a seal of high resistance (> 10GΩ) could be formed between the patch pipette and cell membrane.

After formation of a cell-attached patch, 5-HT (serotonin creatinine sulphate, Sigma) could be applied to the cell by microperfusion from a leaky pipette with a tip of approximately 10 to 20μm in diameter. The microperfusion pipette was filled with 10^{-4}M or 10^{-6}M 5-HT in saline and lowered into the solution near the cell, using a Prior micromanipulator. Tests with Fast Green dye showed that this method was sufficient to bathe the cells in a test solution of drug.

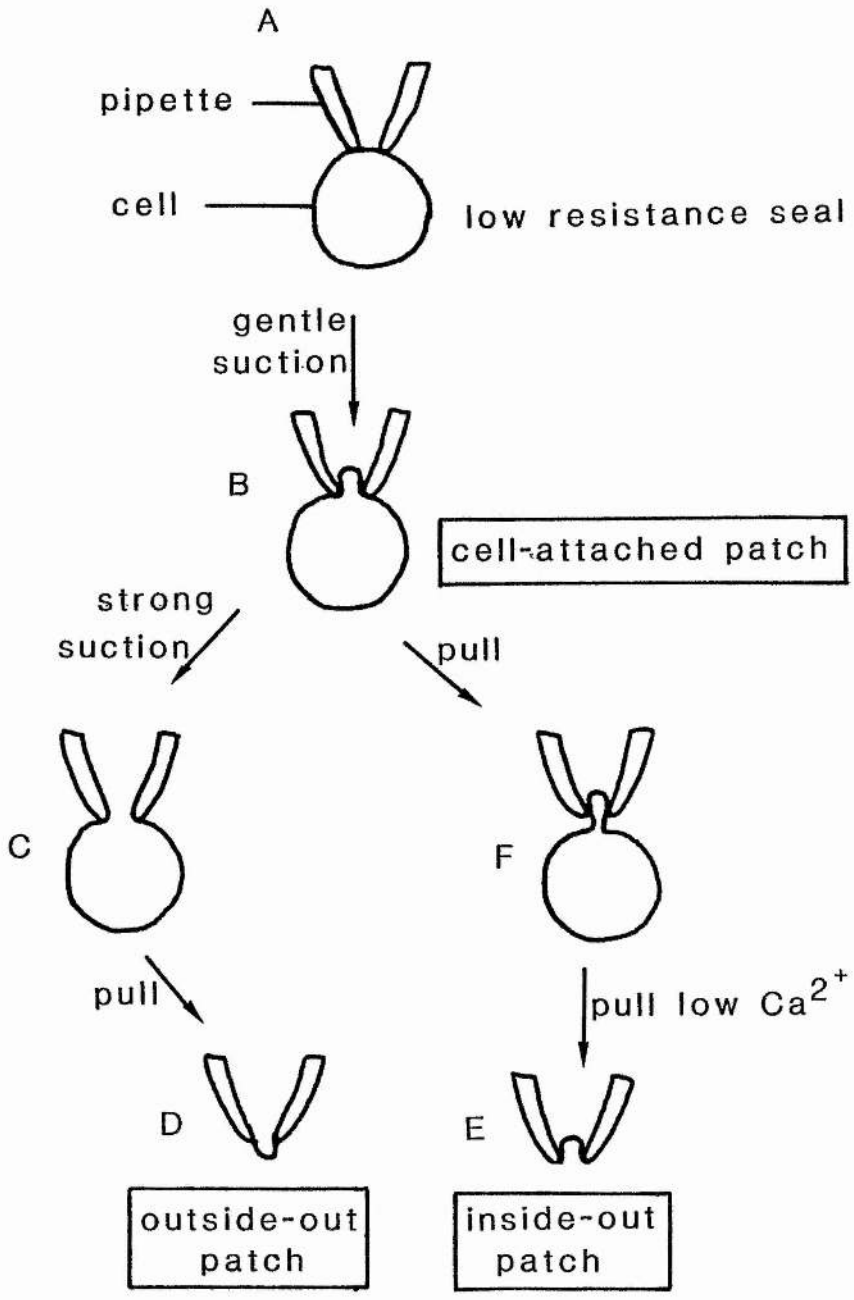
2.3.5 ISOLATED PATCHES

Isolated membrane patches could be formed as described by Hamill et al (1981). Inside-out patches were obtained by moving the pipette away from the cell once a cell-attached patch had formed. The binding of the cell membrane to the glass of the patch pipette, creating the high resistance seal, means that the patch of membrane remains attached to the glass when removed from the cell. Formation of inside-out patches was performed in nominally zero Ca solution, since in the presence of Ca^{2+} ions, the membrane tended to reseal to form a vesicle. However, if a vesicle did form, it could be disrupted by bringing the patch pipette out to the air for a second.

Isolated outside-out membrane patches were formed by applying suction after the formation of a cell-attached patch. This caused the membrane under the patch to disrupt, giving access to the interior of the cell. With the correct solutions in the patch pipette (high K and low Ca) and normal extracellular solution in the bath, removing the patch pipette usually resulted in the formation of an isolated outside-out patch of membrane. A diagram illustrating the formation of the different configurations of membrane patches is shown in figure 2.7.

Figure 2.7

An illustration of the different configurations of the patch-clamp technique (after Hamill et al, 1981). A, formation of a low resistance seal (about 50MΩ) between the pipette and cell membrane by the advancement of the pipette onto the cell. B, formation of a high resistance seal (> 10GΩ) by applying gentle suction to the pipette. This represents the cell-attached configuration. C, strong suction disrupts the membrane under the pipette. D, subsequent pulling of the pipette from the cell causes a portion of membrane to remain attached to the pipette. This reseals to form an outside-out patch. E and F, after formation of a cell-attached patch, pulling away the pipette may result in the formation of an inside-out patch (F) when the preparation is bathed in a low Ca solution.


A
pipette
cell
low resistance seal
gentle suction
B
cell-attached patch
strong suction
pull
C
F
pull
pull low Ca^{2+}
D
E
outside-out patch
inside-out patch

2.3.6 PERFUSING ISOLATED PATCHES

Using the six-way tap described previously, isolated patches of membrane could be perfused with different solutions. A microperfusion chamber was connected to the inflow of the bath. This chamber was constructed from a glass capillary tube, the end of which was sealed by melting in a Bunsen flame. Air from a syringe was blown through the molten tip to create a bulb shaped chamber of 2mm in diameter (see figure 2.5). This microperfusion chamber was similar to, but larger than, that described by Barrett, Magleby and Pallotta (1982). The patch pipette, containing the isolated patch of membrane, could be positioned in this chamber, to facilitate the rapid exchange of solution at this site. While perfusing isolated patches, the microperfusion chamber was kept near to the top of the bath solution to minimise contamination from the bath. Solutions used in isolated patch experiments are listed in table 2.1.

2.3.7 DATA ANALYSIS

Single channel current records were stored on magnetic tape for later analysis. Recordings were played back onto a Nicolet 3091 digital oscilloscope, on which channel amplitudes could be measured by eye, using the movable cursors of the oscilloscope. Automated measurements of channel amplitudes were also performed, using an amplitude program written for the BBC model B microcomputer, and utilising a Unilab 8-bit interface to convert

analogue signals to digital (Cottrell, Duff, Dunbar and Green, 1985). This program operated by the setting of a baseline at the level of the closed channel and a threshold level just above the baseline noise. A length threshold could also be set to avoid problems with brief events which have been attenuated by the filtering of the recording system. The length threshold in the experiments described here was usually 2ms. The program compared the means of four consecutive data points with a previously calculated mean to detect a change in amplitude level. If the signal continued at a new level for longer than the length threshold, it was considered a new amplitude. Amplitude histograms were constructed and a calibration grid added. A print-out of the histogram was obtained on an Epson MX 80 printer.

The measurements of open and closed times of single channel currents also utilised a program for the BBC microcomputer, coupled to a Unilab interface (Cottrell, Duff and Lambert, 1983). This program set cursors at the base and peak levels, as well as lower and upper windows, which were normally spaced at 40% and 60% of the distance between the base and peak levels. Events which crossed, first the lower, then upper windows were considered to be openings and similarly, events crossing the upper, then lower windows were considered to be closings. Thus the durations of times spent in the open and closed states could be measured. The raw data of open and closed times were stored on floppy disc and a print-out of the total and mean open and closed times was given.

Values of open and closed times, stored on floppy disc, could be transferred to a Vax VMS computer on which statistical analysis, using the "Genstat" package, enabled exponential distributions to be fitted to the open and closed times. This statistical analysis was performed on records presumed to contain only one channel (that is records in which openings to a second level were not observed). "Genstat" programs (written by D. Sinclair and J. Newton, Dept. of Statistics, University of St. Andrews), used the method of maximum likelihood to fit exponential distributions to frequency distribution histograms of open and closed times.

2.4 VOLTAGE CLAMP METHODS

2.4.1 RECORDING ELECTRODES

Electrodes for intracellular recording were made from filamented capillary glass (Clark Electromedical Instruments cat. no. GC 150-TF-15), and were pulled on a home made vertical electrode puller. Electrodes were filled with 2M K acetate or 1M KCl and were pulled to have a resistance of 1 to 3MΩ when filled with these solutions. The presence of the filament within the glass facilitated filling of the solution to the tip of the electrode, by capillary action. The remainder of the electrode was filled using a syringe and long hypodermic needle or thinly tapered polythene tubing.

2.4.2 SINGLE ELECTRODE VOLTAGE CLAMP

Voltage clamp recordings, not requiring a high frequency response, or the passage of large currents (> 30nA), were performed using a Dagan 8100 single electrode voltage clamp system. The Dagan 8100, in the switched clamp mode, operates by a technique similar to that described by Wilson and Goldner (1975). This system permits current passing and voltage sensing with the same electrode, whilst overcoming the problems of changes in electrode resistance and non-linearity of electrode resistance. It achieves this by utilising an electronic switch, to switch at high frequencies from current passing to voltage sensing modes. The frequency of switching in experiments described here was 3KHz and the duty cycle was 50%, meaning that half of the time was spent in each mode. The gain of the feedback amplifier for voltage clamping was set as near to maximum as possible, without causing the system to oscillate. Lower resistance electrodes enabled the gain to be set near to the maximum.

The recording chamber was grounded by a Ag/AgCl wire (or indirectly by an agar bridge) to the virtual ground of the Dagan probe (a high input impedance amplifier). 2M K acetate electrodes were connected, via a Ag/AgCl wire to the input of the probe, and shielded by a metal spring connected to the driven shield of the probe.

Recordings of current and voltage were monitored on a Tektronix 502A or 561A oscilloscope and permanent records made on a high quality brush chart recorder (Gould 220 series). The current input to the chart recorder first passed through a low pass filter, consisting of a 160KΩ resistor in series with a 10^{-7}F capacitor, producing filtering at 10Hz. This reduced the high frequency noise, but did not affect the slow responses studied.

2.4.3 IONOPHORESIS

Ionophoretic application of 5-HT and intracellular ionophoresis were performed using a WPI microiontophoresis programmer (model 160). Ionophoretic electrodes were pulled to the same size as K acetate recording electrodes. 5-HT ionophoretic electrodes were filled with a solution of 10mg/ml 5-HT in distilled water. The reference electrode for ionophoresis was usually a Ag/AgCl wire in the bath or a K acetate electrode positioned a small distance from the ionophoretic electrode. To apply 5-HT, ejection currents of 20 to 40nA, for a duration of 4s, were applied. A retain current of 10nA was used.

Intracellular ionophoresis of Ca, EGTA or cAMP was performed from pipettes containing either 0.5M $CaCl_2$ in distilled water, 0.5M EGTA, dissolved in 1M KOH to pH 7.0 or 0.1M cAMP dissolved in 0.1M KOH, to pH 7.0. Ionophoresis into cells was either from doubled barrelled electrodes (with both barrels containing the same solution in order to minimise any pH effects from the injection of

OH^- or H^+ ions) or from a single electrode, with the cell held under voltage clamp conditions. The ejection currents and durations shall be described in the results section for individual experiments.

2.3.5 DOUBLE ELECTRODE VOLTAGE CLAMP

Experiments involving stepping Vm from -50mV to potentials up to +170mV were performed using a home-made fast microelectrode voltage clamp amplifier (based on the design of Lee, Akaike and Brown, 1980). This system could operate as either a single or double electrode clamp. It was used in the double electrode mode in these experiments, to minimise series resistance problems encountered with single microelectrode systems. The recording and current passing electrodes were both filled with 1M KCl. It was important to keep the resistance of the current passing electrode as small as possible (usually $<2M\Omega$), in order to be able to pass sufficient current to clamp the cells to the very positive potentials required here. The bath was grounded to the ground input of the amplifier via an agar bridge. After impaling the cell, and changing the amplifier to the voltage clamp mode, the variable gain of the amplifier was increased as high as possible, without causing the system to oscillate.

Although a double electrode voltage clamp was used, series resistance across the bath and agar bridge may induce errors, since the currents passed were very large (up to 3μA). The resistance of the agar bridge was calculated to be 2KΩ. With a current of 5μA,

this would produce a 10 mV error in the voltage clamp. To minimise this error, a series resistance compensation circuit was included in the amplifier. Increasing the series resistance compensation to just below the point of oscillation should minimise the errors in the voltage recording (W. Stevens, personal communication).

A Tektronix type 162 waveform generator and type 161 pulse generator were used to supply a 20V pulse to the amplifier. The stimulus attenuation control of the amplifier could then be used to regulate the amplitude of the voltage step. Steps of 100ms duration were applied with a 15s interval between steps. Currents, digitised by a Sony digital audio processor (PCM-701 ES), were recorded on a JVC VHS video recorder and monitored on a Tektronix dual beam storage oscilloscope. Measurements of currents were made on a Nicolet digital oscilloscope, utilising the movable cursors. Hard copies of selected traces were made by taking photographs from the oscilloscope screen, using a Polaroid camera.

CHAPTER 3

RESULTS

RESULTS

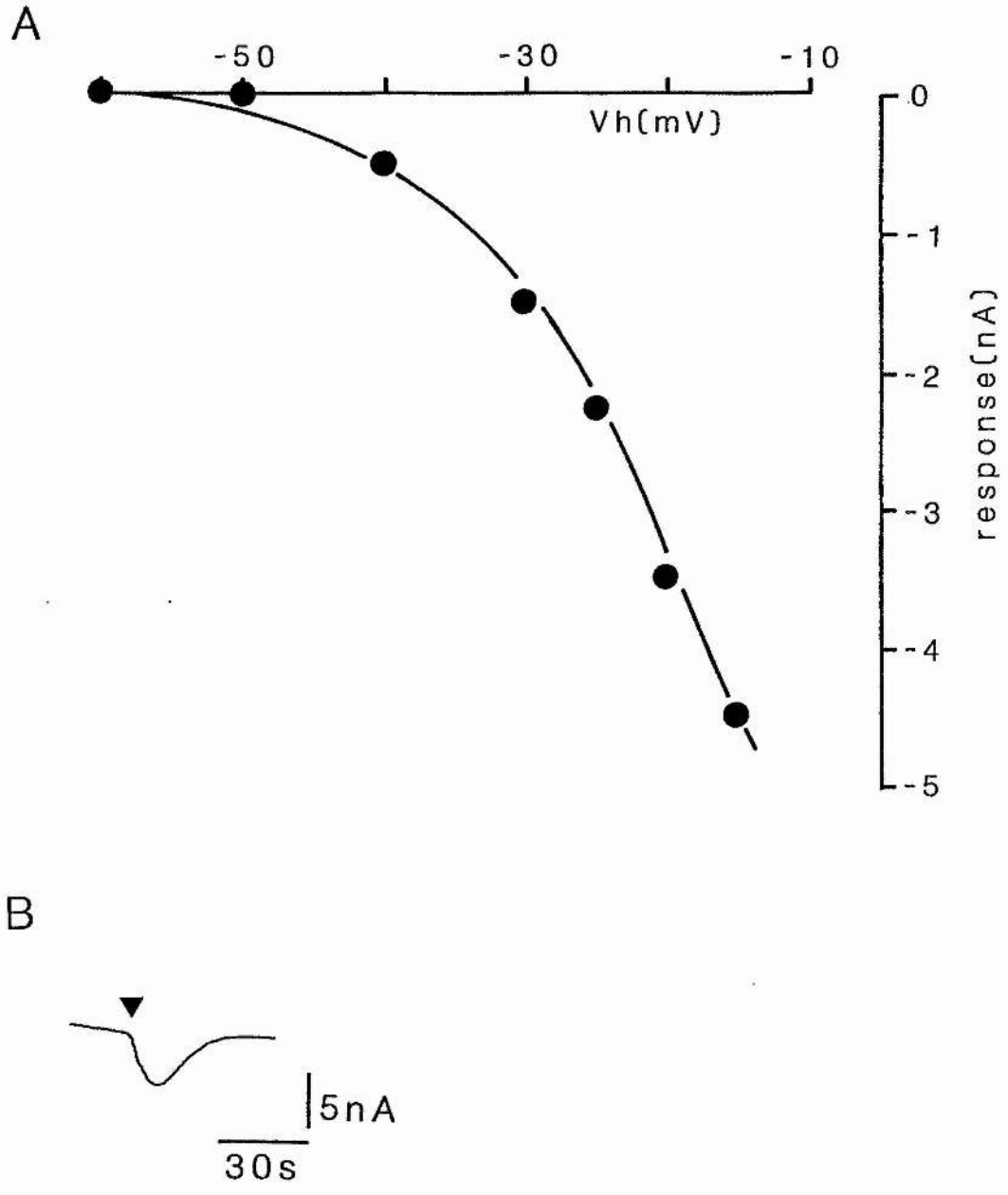
The results described in this thesis are from a voltage clamp and patch clamp study of the 5-HT response in the C1 neurone of Helix aspersa. The patch clamp technique enabled an investigation of the activity of K channels present in the C1 neurone, and the modulation of these channels by 5-HT. Voltage clamp techniques were used to investigate the Ca-dependency of the response and the possible role of cAMP as an intracellular messenger. The ease with which the C1 neurone could be cleanly exposed and identified made it a suitable preparation for such a study. The possible physiological relevance of this 5-HT response, in a known 5-HT containing neurone, made its investigation of added interest. The symmetrically located C1 neurones have been shown to make synaptic connections with each other. Stimulation of one C1 neurone induces an inward current in the contralateral C1 neurone when it is voltage-clamped to a depolarised potential. This action is probably due to the release of 5-HT from the C1 neurone (Cottrell, unpublished observations).

3.1 THE VOLTAGE-DEPENDENT 5-HT RESPONSE OF THE C1 NEURONE

When the C1 neurone was held under voltage clamp conditions, ionophoretic application of 5-HT induced an inward current response which showed marked voltage sensitivity. Figure 3.1 shows the relationship between response amplitude and holding potential recorded from a C1 neurone using a single electrode voltage clamp technique. Figure 3.1,B is an example of an inward current response to

Figure 3.1

The inward current response to 5-HT in a voltage-clamped C1 neurone. A, relationship between response amplitude and holding potential. B, an example of an inward current response to ionophoretically applied 5-HT (▼) in a C1 neurone at a holding potential of -20mV.


A
-50
-30
-10
Vh(mV)
0
-1
-2
-3
-4
-5
response(nA)
B
5nA
30s

ionophoresed 5-HT. The response to 5-HT was very small, or absent at holding potentials more negative than -50mV and was never observed to reverse with further hyperpolarisation. Voltage clamping the C1 neurone to more depolarised potentials caused a marked increase in the size of the 5-HT response, as seen in figure 3.1,A. This response has previously been shown not to depend on Na^+ and Cl^- ions, but it was sensitive to the inorganic Ca channel blocking agents, Co and Cd, at concentrations of 1 to 10mM (Cottrell, 1982b; Barnes, Cottrell and Dunbar, in preparation). The sensitivity to Ca channel blocking agents suggested that the response could be due to an increase in a voltage-dependent gCa or a decrease in a Ca-dependent gK, activated by Ca entry during depolarisation. Earlier experiments have also shown that the 5-HT response is sensitive to changes in the extracellular K concentration. Raising the K concentration from 5 to 35mM caused a reduction in the amplitude of the response, as would be expected if the response was due to a decrease in gK. Ba^{2+} ions, which are known to carry Ca currents, but block K currents (Hermann and Gorman, 1979), completely abolished the voltage-dependent 5-HT response in the C1 neurone, suggesting that it is indeed due to a decrease in gK (Barnes, Cottrell and Dunbar, in preparation).

The patch clamp technique has been utilised in the investigation of a similar 5-HT response in *Aplysia* sensory neurones (Siegelbaum *et al*, 1982). A patch clamp investigation of the K channels present in the C1 neurone was undertaken and a study made of the effect of 5-HT on the channels recorded. It was hoped that the patch clamp technique would be useful in determining the Ca-dependency of the K channels modulated by 5-HT. The following sections describe some observations

made on outward single channel currents recorded in the C1 neurone, the characterisation of these single channel currents and the effect of 5-HT.

3.2 SINGLE CHANNEL CURRENTS IN CELL-ATTACHED PATCHES OF THE C1 NEURONE

Patch clamp recordings were made with normal extracellular solution in both the bath and patch pipette. Generally, after formation of the high resistance GΩ seal, no single channel currents were observed at the resting potential. The patch of membrane had to be depolarised before unitary currents could be observed. In the cell-attached mode, making the inside of the patch pipette more negative was equivalent to depolarising the patch of membrane. The term patch potential (Vp) is used throughout to refer to the potential across the patch of membrane. The patch potential was calculated by subtracting the potential inside the pipette (Vpipette) from the membrane potential of the cell (Vm). This is shown in equation 3.1:

$$Vp = Vm - Vpipette. \qquad (3.1).$$

Thus with Vm = -50mV and Vpipette = -50mV, the potential across the patch of membrane (Vp) is 0mV. In some experiments, in order to calculate Vp, Vm was recorded with an intracellular electrode during patch recordings. In other experiments, when possible, an attempt was made to measure Vm at the end of the experiment by rupturing the membrane under the pipette. This gave access to the interior of the cell, and Vm could then be measured using the current clamp mode on the patch amplifier. When no record of Vm could be obtained, Vm was

assumed to be -60mV, which was the average value of Vm calculated from 38 recordings from the C1 neurone using both intracellular and patch electrodes (mean = -59.8±8.0mV). All values are quoted as the mean± the standard deviation.

On depolarising the patch of membrane, single channel outward currents became evident. Two sizes of outward channel currents were commonly observed on cell-attached patches of the C1 neurone. Figure 3.2 shows a section of recording from a C1 neurone (Vp=+30mV) in which outward channel currents of two different amplitudes are present. Both types of channel were not observed in all patches of the C1 neurone. In some patches only one type was observed, but frequently they occurred together. However, if the larger single channels were very active this often made it difficult to see and measure the much smaller single channel events.

In some records more than two sizes of unitary currents were observed. However, intermediate sizes were only rarely seen, and sufficient records to construct an I-V relationship were not obtained. Figure 3.3 is an example of a record in which three sizes of unitary currents were observed. Since intermediate sized channels were observed so rarely on patches of the C1 neurone, no further analysis of them was made.

Another observation from recordings of channels in cell-attached patches, was an infrequently occurring subconductance state of the large unitary currents. An example of such a substate is shown in figure 3.4. In this record no inward events were observed. Therefore,

Figure 3.2

Two sizes of outward channel current commonly observed in cell-attached patches of the C1 neurone. This record was filtered at 1KHz. Vp=+30mV.

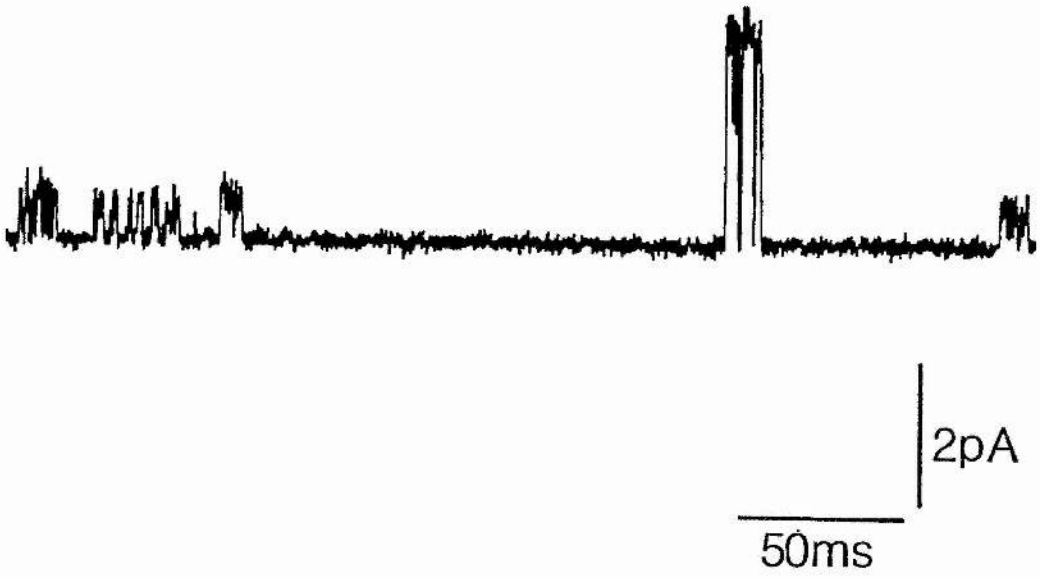

2pA
50ms

Figure 3.3

Three different sizes of outward channel currents recorded from a cell-attached patch of the C1 neurone at a patch potential of about +20mV. Vm was assumed to be -60mV in this record. The upper trace shows small unitary currents of about 0.8pA, corresponding to the small channels usually observed on cell-attached patches. The middle trace shows an intermediate size of about 1.9pA, not normally observed at this potential, while the lower trace shows channels of about 3pA, corresponding to the large unitary currents usually observed in cell-attached patches of the C1 neurone. This record was filtered at 2.5KHz.

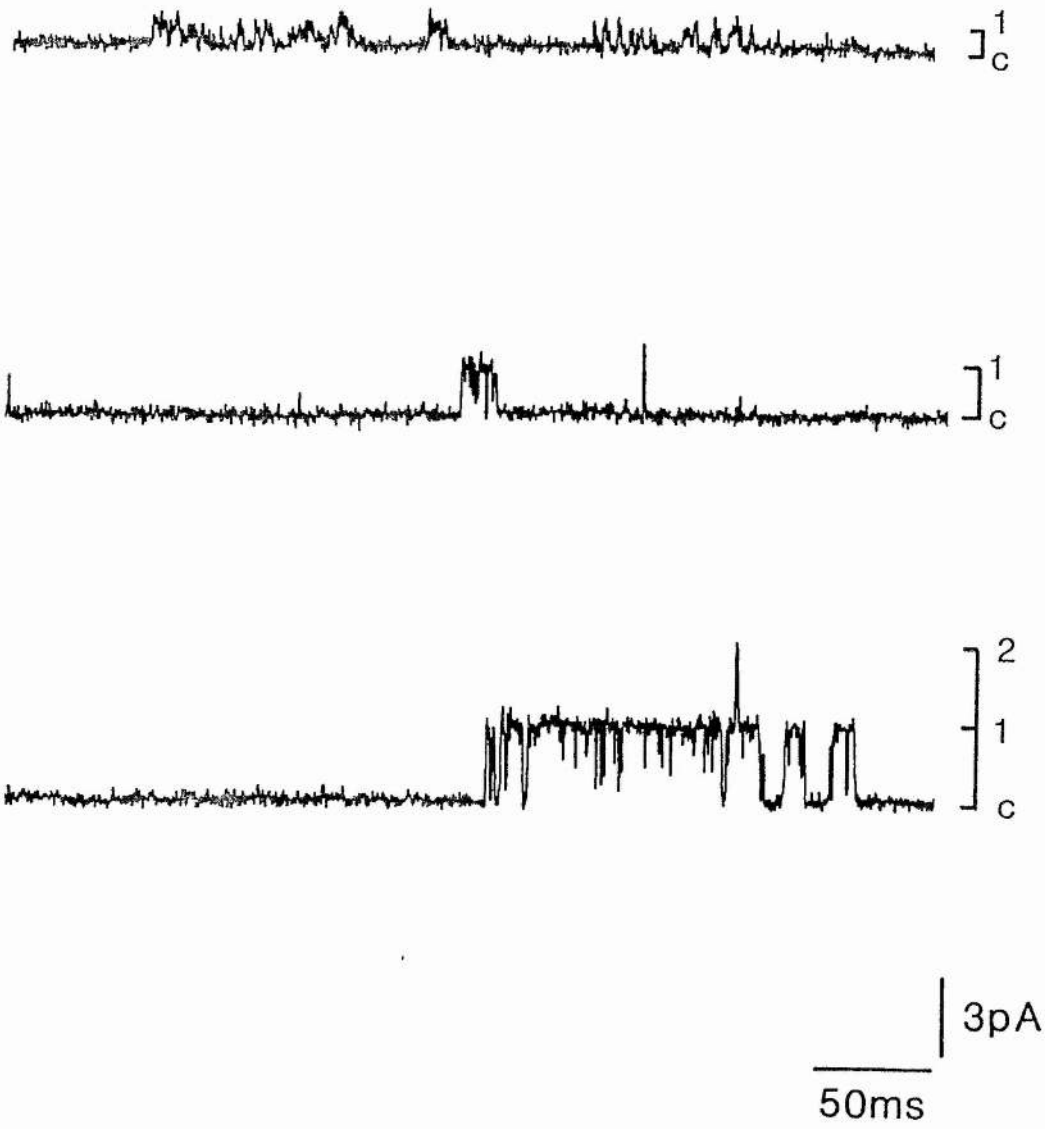
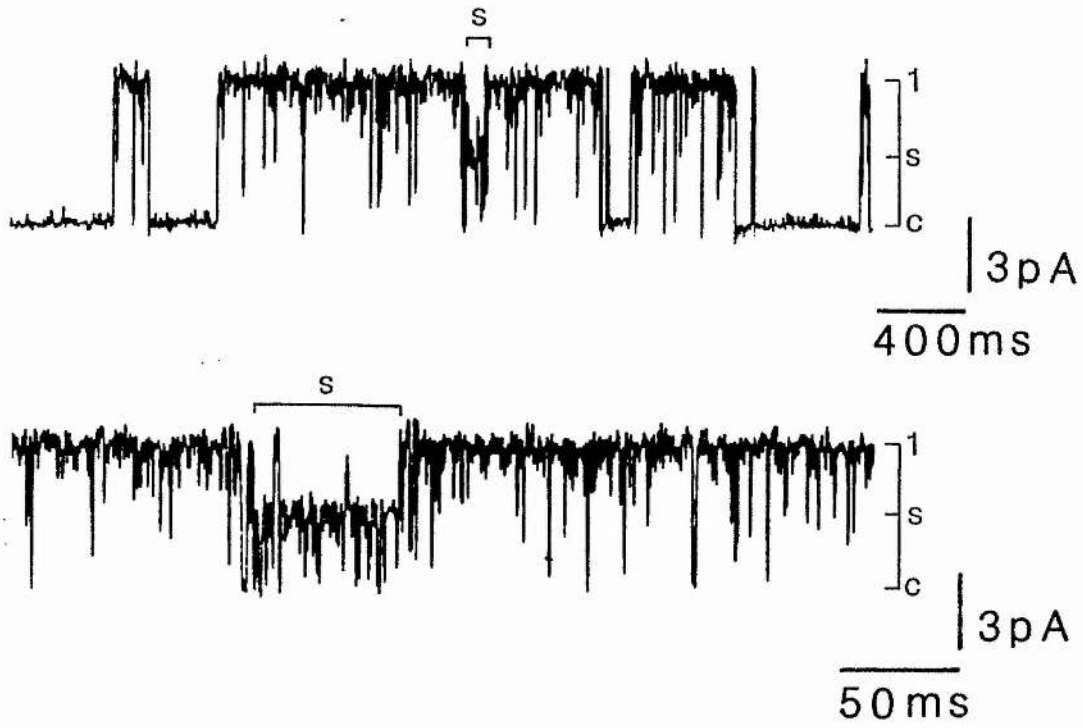

1
c
1
c
2
1
c
3pA
50ms

Figure 3.4

An infrequently observed subconductance state of the larger single channel outward currents. The lower trace shows a section of the upper trace on an expanded time scale. The subconductance state is marked S in both records. Closed, fully open and substates of the channel are marked on the right side by , C, 1 and S respectively. Recordings were filtered at 1KHz. Vp=+59mV.


s
1
s
c
3pA
400ms
s
1
s
c
3pA
50ms

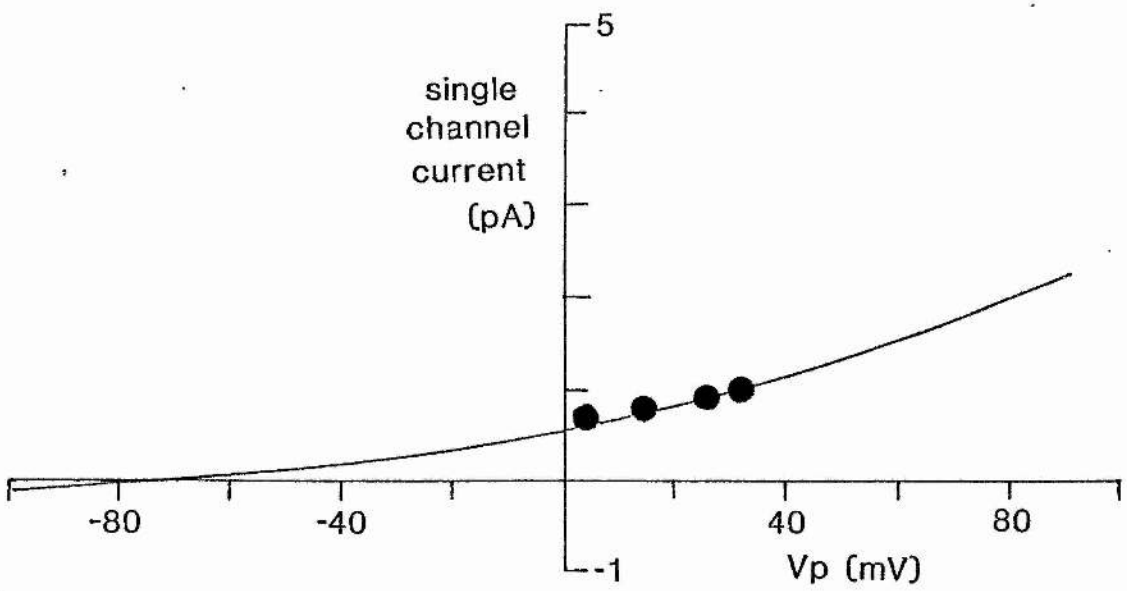
this substate was unlikely to be accounted for by the simultaneous occurrence of inward and outward events. Similarly, no openings of channels directly to the level of the substate were observed. The substate level appeared (within the limits of resolution) to be reached from the fully open state of the large outward channel current.

3.2.1 SMALL UNITARY OUTWARD CURRENTS

When possible, I-V relationships of the smaller unitary outward currents were constructed. The smaller unitary outward currents were less frequently observed than the larger ones. The low conductance of these channels made accurate measurements of their amplitude more difficult than for the larger channels. Furthermore, the presence of the large unitary currents often obscured these much smaller unitary currents. An I-V relationship of small unitary outward currents recorded from a cell-attached patch is shown in figure 3.5. Generally the I-V relationship could be fitted well with the theoretical relationship calculated from the Goldman-Hodgkin-Katz (GHK) equation for a K current. The GHK constant field current equation was derived by Hodgkin and Katz (1949) and was based on the constant field theory for membrane rectification (Goldman, 1943). This derivation assumed a constant electric field throughout the membrane, an independent movement of ions and a homogeneous membrane. The latter assumption is clearly incorrect, since it is now known that ions move through channels within the membrane. The current equation derived by Hodgkin and Katz (1949) was for the current density across a cm^2 area of membrane and, hence, the units of permeability were $cm.s^{-1}$. However, in the equations used here, the absolute current through the channels

Figure 3.5

An I-V relationship of the smaller outward channel currents recorded from a cell-attached patch. The line drawn through the points represents the theoretical relationship based on the GHK equation for a K current. The permeability value (P_K=6.39x10^{-14}cm^3.s^{-1}) was calculated from the data points, assuming an intracellular K concentration of 98mM. The resting membrane potential recorded with the patch pipette at the end of this recording was -57mV. The slope conductance of these channels was measured to be 13pS at a Vp of +10mV.


5
single
channel
current
(pA)
-80
-40
40
80
-1
Vp (mV)

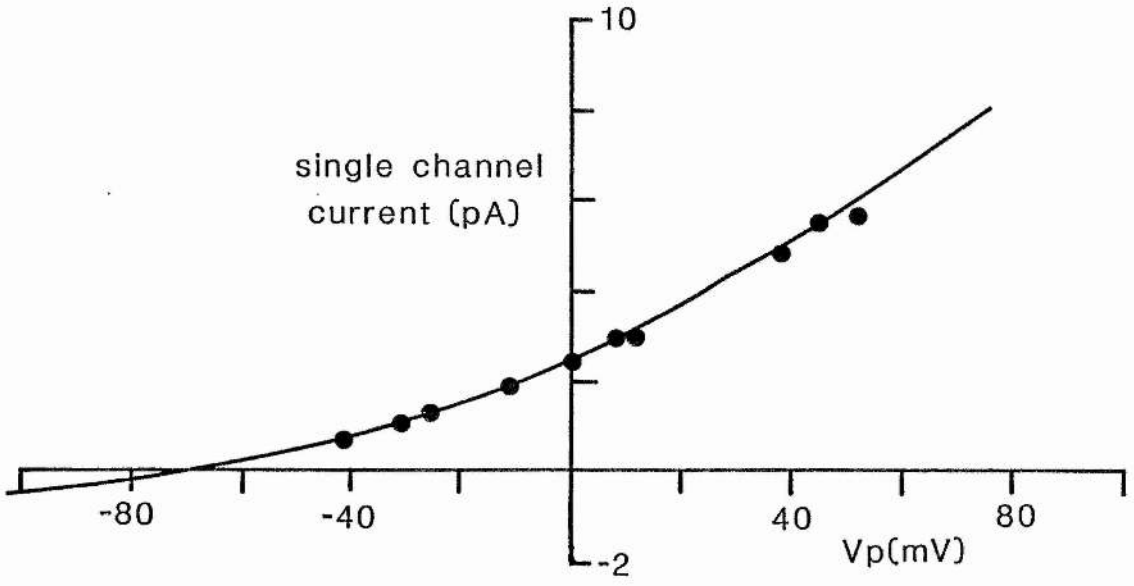
was used, and the absolute permeability calculated. The units of permeability are therefore $cm.^3s^{-1}$. Fits to the GHK equation were made assuming an intracellular K concentration of 98mM (Alvarez-leefmans and Gamino, 1982; Cottrell, Davies and Green, 1984). The value of permeability (P_K) used was the mean value calculated from the data points. In this example $P_K = 6.39x10^{-14} cm.^3s^{-1}$. The mean P_K value calculated for these channels was $6.7 \pm 1.4x10^{-14} cm.^3s^{-1}$ (n=18). The slope conductance of unitary currents at Vp=+10mV was used as a means of identifying and comparing the single channel currents. To measure slope conductance a tangent to the curve was drawn at +10mV (Vp) and the gradient of this line calculated. In the example in figure 3.5 the slope conductance was 13pS. The mean slope conductance of these channels at +10mV was 14.2 ± 2.1pS (n=6) and the mean amplitude of the channels was 0.73 ± 0.11pA.

3.2.2 LARGE UNITARY OUTWARD CURRENTS

More accurate measurements of the larger unitary outward currents could be obtained due to the favourable signal to noise ratio for these channels at depolarised potentials. The I-V relationship of these single channel currents was investigated. Figure 3.6 shows the I-V curve of large unitary currents from a cell-attached patch in which Vm was simultaneously measured with an intracellular electrode. The relationship of single channel current to Vp could be fitted well with the theoretical relationship calculated from the GHK equation for a K current. The permeability value calculated from the data points was $2.71x10^{-13} cm.^3s^{-1}$. The mean permeability value calculated for the larger unitary currents was $2.3 \pm 0.4x10^{-13} cm.^3s^{-1}$ (n=20). All values

Figure 3.6

An I-V relationship of the larger single channel outward currents, recorded from a cell-attached patch of the C1 neurone, whilst simultaneously recording membrane potential with an intracellular electrode. The resting potential of this cell was measured as -70mV. The I-V relationship of these channels was non-linear, showing rectification, which agreed closely with the theoretical relationship, calculated from the GHK equation for a K current (solid line). The permeability value (P_K= $2.71 \times 10^{-13} cm^3.s^{-1}$) was calculated, assuming an intracellular K concentration of 98mM. In this example the slope conductance was measured to be 57pS at a Vp of +10mV.


10
single channel
current (pA)
-80
-40
40
80
Vp(mV)
-2

for the means are quoted $\pm$ the standard deviation. The slope conductance of the channels shown in figure 3.6 was 57pS at Vp=+10mV. The mean slope conductance at +10mV, from six experiments was 53.8 ± 3.3pS and the mean channel amplitude at this potential was 2.5 ± 0.3pA. The terms 54pS and 14pS channels shall be used to refer to the large and small channels recorded in cell-attached patches of the C1 neurone.

The fact that the I-V relationships of both small and large unitary outward currents agreed well with the theoretical relationship, according to the GHK equation for a K current, suggested that the charge carrying species of these unitary outward currents was K^+ ions. The GHK equation is based on the constant field theory for the rectification of current flow through cell membranes (Hodgkin and Katz, 1949). In figures 3.5 and 3.6 it can be seen that the I-V relationships of both types of channels are non-linear. Reversal of current flow through these channels was never observed in cell-attached patches. No unitary currents were observed at hyperpolarised patch potentials.

The absence of unitary outward currents at hyperpolarised potentials could be due to a voltage-dependent gating of the ion channels. Alternatively, it could be explained by a rectification of the current flow through the channels, such as the rectification described by the constant field theory. This predicts that the amplitude of the K current will be much smaller in the hyperpolarised direction than in the depolarised direction. Small unitary currents may then be lost amongst the background noise, thereby preventing their

detection. Unitary currents were generally not observed in cell-attached patches at potentials less depolarised than -30mV. The agreement of the unitary current data with the constant field theory for a K current gave indirect evidence that K^+ ions carried the charge through these channels. This theory was tested directly by examining the dependence of the unitary currents on the K concentration.

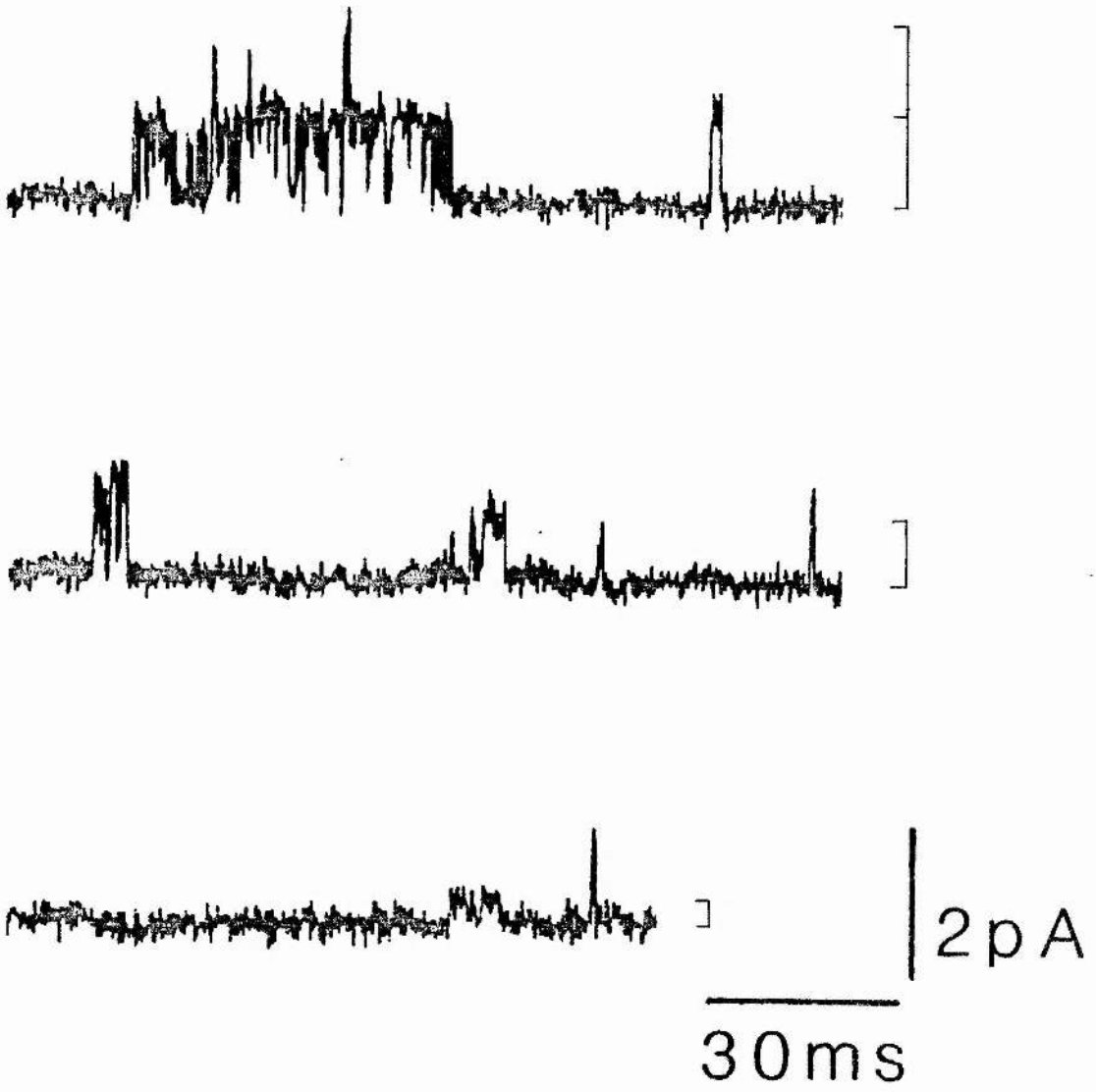
3.3 INCREASING THE K CONCENTRATION OF THE PIPETTE

Recordings were made from cell-attached patches of the C1 neurone, using a high K solution in the patch pipette. This solution contained 35mM K instead of the usual 5mM K, and was made by substituting 30mM NaCl with KCl. Raising the K concentration on the external surface of the membrane would be expected to reduce the driving force for the outward movement of K^+ ions, thereby reducing a current which was due to the flow of K^+ ions. In these experiments the cell was bathed in normal solution, so avoiding a depolarising effect of the high K solution. Results from these experiments suggested that the unitary currents observed were carried by K^+ ions.

Outward channel currents, recorded with high K solution in the patch pipette, were smaller than currents normally recorded in control conditions. Figure 3.7 shows some unitary outward currents recorded from a cell-attached patch, with 35mM K^+ ions in the patch pipette. This recording was made at a patch potential of +10mV. Three different amplitudes of single channel currents appeared to be present in this record. However, it is possible that the largest amplitude observed here was due to the simultaneous opening of the other channels. The

Figure 3.7

Single channel outward currents recorded from a cell-attached patch of the C1 neurone, with a high K solution (35mM) in the patch pipette. The appearance of three different amplitudes in this record is marked on the right of the traces. Vp=+10mV. Records were filtered at 2.5KHz.


2pA
30ms

amplitudes of single channel currents observed under these conditions were 1.25, 0.75 and 0.35pA. These amplitudes compare with values of 2.5 and 0.73pA, for the 54pS and 14pS channels respectively, with normal solution in the patch pipette. However, due to the nature of the experiment, it was not possible to obtain control recordings from the same patch since there was no facility for perfusing the patch pipette. The permeability values of the unitary current recorded in high K were lower than in control conditions. Values of P_K were $1.4x10^{-13}$, $8.6x10^{-14}$ and $4.0x10^{-14}cm.^3s^{-1}$, for the 1.25, 0.75 and 0.35pA currents respectively. No I-V relationships for unitary currents with high K in the patch pipette were obtained. The presence of different types of channels with smaller unitary currents with high K in the patch pipette, suggested that the outward channel currents observed on cell-attached patches were carried by K^+ ions. More conclusive evidence for this was obtained from experiments on isolated outside-out membrane patches.

3.4 PERFUSED OUTSIDE-OUT PATCHES

Experiments on isolated outside-out membrane patches allowed the outer surface of the patch of membrane to be perfused with different solutions. Perfusion of isolated patches was achieved using the microperfusion chamber described on page 75 of the methods section. The pipette containing the isolated patch of membrane could be transferred to this chamber to enable a rapid exchange of solution in the area of the patch. Outside-out patches were formed with a 96mM K solution in the patch pipette (solution 2, Table 2.1). Recordings from isolated outside-out patches were made with normal 5mM K solution on

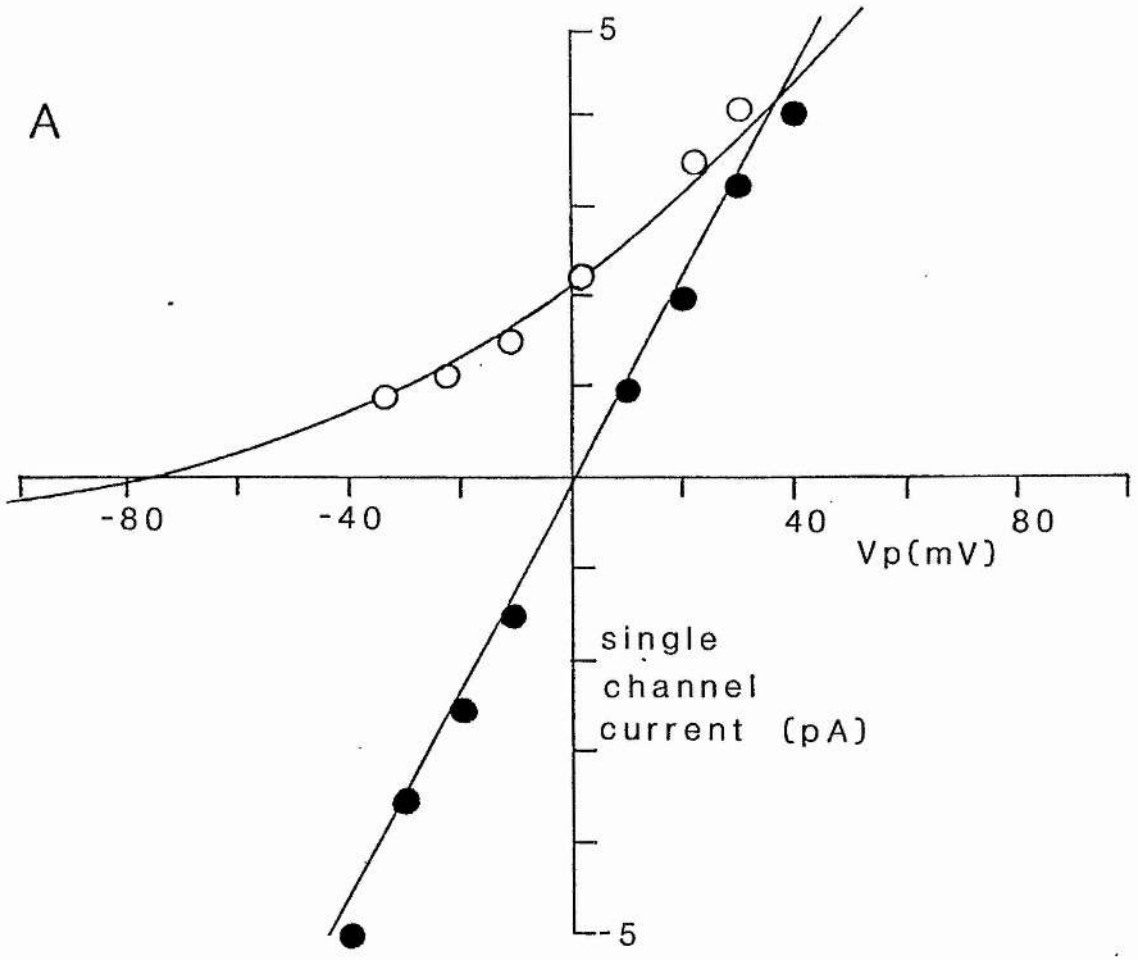
the external surface or with 96mM K solution or 44mM K solution (solutions 2 and 3, table 2.1) perfusing the patch of membrane. The 44mM K solution was made by substitution with sucrose to maintain the osmolarity. Figure 3.8 shows examples of I-V relationships for the large unitary outward currents with 5 and 96mM K or 44 and 96mM K on the external surface. In general the larger channels were very active in isolated patches but the smaller channels were not observed, or were obscured by the larger channels.

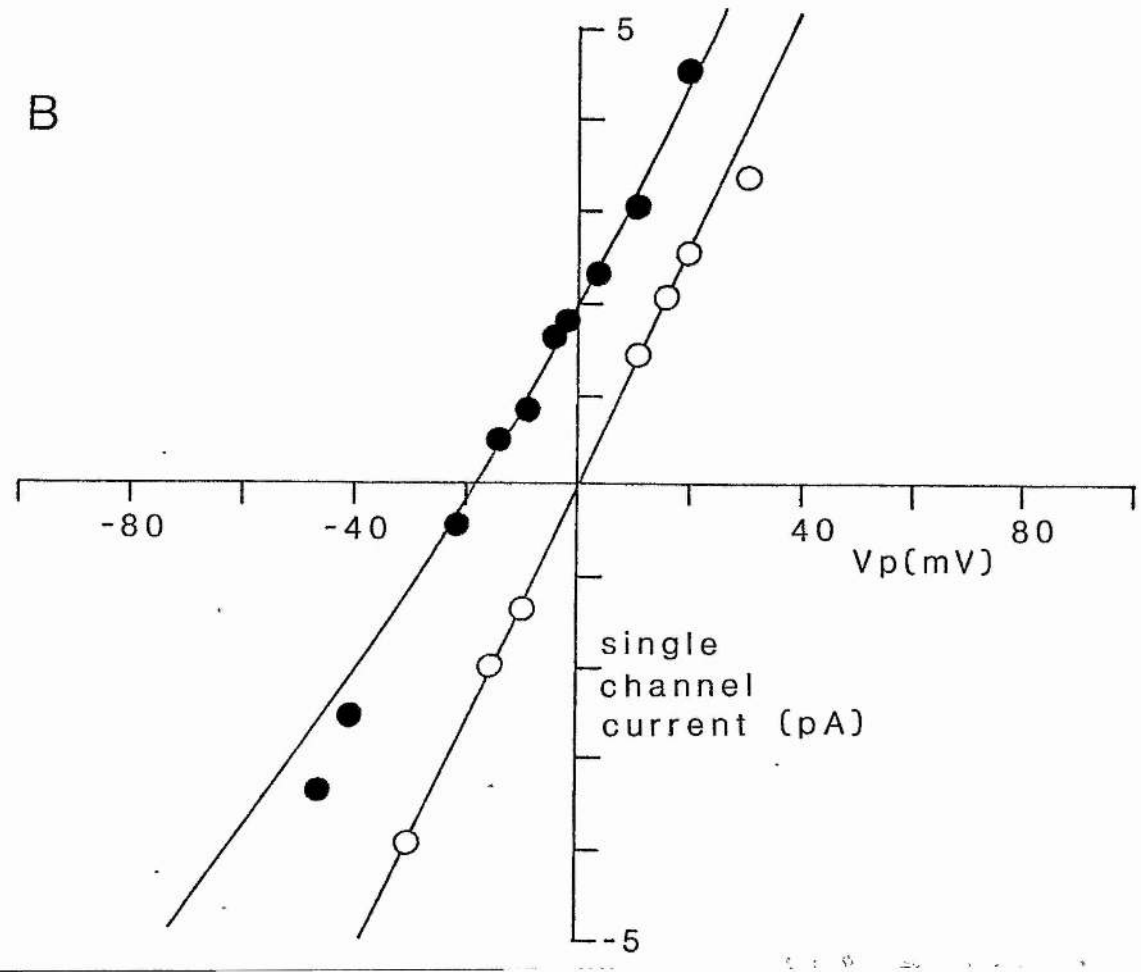
Attempts to obtain recordings from the same patch in all three solutions were not successful and the records shown are from patches perfused with two of the solutions. With 96mM K on the inner surface and 5mM K on the outer surface (conditions resembling those in a cell-attached patch), the I-V curve of the channels displayed pronounced rectification. Under these conditions no clear reversal of the single channel current was observed (figure 3.8,B). As with recordings from cell-attached patches, the I-V relationship of the channels under these conditions could be fitted by the theoretical relationship of the GHK equation for a K current, allowing the reversal potential to be extrapolated from this fit. In symmetrical 96/96mM K solution the I-V relationship was linear, reversing at 0mV. The conductance value of the channels in symmetrical solution was between 130 and 170pS (figure 3.8,A and B). In 96/44mM K solutions the I-V curve displayed slight rectification. The mean reversal potential of the unitary currents under these conditions was -19.6 ± 1.0mV in four experiments. In the example in figure 3.8,B the conductance value at +10mV was about 125pS. In all three solutions the I-V relationships could be fitted well by the GHK equation and, therefore the reversal

Figure 3.8

Isolated outside-out membrane patches perfused with different K concentrations. In all records the patch pipette contained 96mM K. A, I-V relationship of single channel outward currents recorded from an outside-out patch with 5mM K (open circles) or 96mM K (filled circles) on the outer surface of the membrane. Both sets of data have been fitted with the theoretical relationship from the GHK equation for a K current, using mean permeability values calculated from the data points. In asymmetrical, 5/96mM K conditions (open circles), P_K was $2.38 \times 10^{-13} cm^3.s^{-1}$ and the slope conductance was measured to be 50pS at a Vp of +10mV. In symmetrical, 96/96mM K solutions, (filled circles), P_K was $3.12 \times 10^{-13} cm^3.s^{-1}$ and the conductance was 130pS.

B, I-V relationships of single channel currents recorded from an outside-out patch perfused with 44mM K (filled circles) and 96mM K (open circles). Fits to GHK equation were made using permeability values calculated from the data points. The permeability value in asymmetrical, 96/44 mM K conditions (filled circles) was $4.17 \times 10^{-13} cm^3.s^{-1}$ and the slope conductance at +10mV was 125pS. The permeability value in symmetrical 96/96mM K solutions (open circles) was $3.56 \times 10^{-13} cm^3.s^{-1}$ in this example and the conductance was 170pS.


A
5
-80
-40
40
80
Vp(mV)
single
channel
current (pA)
-5


B
5
-80
-40
40
80
Vp(mV)
single
channel
current (pA)
-5

potentials of the channels agreed with the predicted values of 0mV, -19.7mV and -74.4mV for the 96mM, 44mM and 5mM K solutions respectively.

The agreement of the shift in reversal potential for these channels with the values calculated from the Nernst equation gave direct evidence that the current flowing through these channels was carried by K^+ ions. Although in each of the solutions the I-V curves could be fitted by the theoretical relationship for the GHK equation, the values calculated from the data for the permeability were different in the different solutions, suggesting that the current flow through the ion channels did not obey the constant field theory assumed in the GHK equation. However, it is known that current flow through many ion channels does not obey the constant field theory for rectification. Shifts in the reversal potentials, which were in agreement with the values calculated from the Nernst equation, were also obtained when a fit by eye was made to the data points. Therefore, the evidence shows that the large unitary outward currents recorded from the C1 neurone are single K channel currents. However, this conclusion is complicated by the later finding of both Ca-dependent and Ca-independent K channels in isolated inside-out patches.

Such direct evidence for the ionic nature of the smaller channels was not obtained, since recordings of these channels in outside-out membrane patches was not obtained. However, the I-V relationship of the 14pS unitary currents suggests that the most likely ionic species involved is K^+ ions. Since the response to 5-HT in the C1 neurone has been found to be due to a decrease in gK, it was of interest to examine

the effect of 5-HT on the K channels recorded in the C1 neurone.

3.5 THE EFFECT OF 5-HT ON K CHANNELS OF THE C1 NEURONE

In order to test the effect of 5-HT on the single channel K currents, 5-HT was applied to the C1 neurone, whilst recording from a cell-attached patch. Using this method, it is believed that 5-HT cannot directly interact with the ion channels under the pipette, due to the high resistance seal formed between the cell membrane and patch pipette. Therefore, any effect which 5-HT exerts on these channels is assumed to be mediated by an intracellular messenger. 5-HT has been found to close K channels in Aplysia sensory neurones by such an indirect means (Siegelbaum et al, 1982).

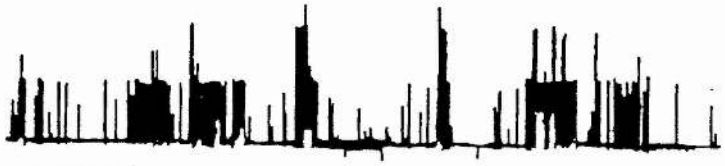
Three different methods of applying 5-HT to the C1 neurone, during recording from a cell-attached patch, were attempted. These included, perfusion of the entire bath, pressure ejection and microperfusion. Perfusing the bath and pressure ejection were found to be less successful, since both of these methods tended to cause a mechanical disruption of the seal. Although it was possible to perform some experiments using these methods, most of the results obtained were from microperfusion experiments. Applying 5-HT by microperfusion involved carefully lowering a leaky pipette containing 5-HT into the solution, close to the C1 neurone. Tests using Fast Green dye showed that this method was sufficient to bathe the cell rapidly in a solution of 5-HT. The major disadvantage of this method was that the effect was not always easily reversed, since it required the 5-HT to diffuse away from the cell.

Microperfusion of 100μM 5-HT onto the C1 neurone, from outwith the patch pipette was found to reduce the total open time of the 54pS K channels, recorded in the cell-attached mode. An example of one such experiment is shown in figure 3.9, where the percentage open time was reduced from 9.8% to 0.4%. A reduction in total channel open time was observed in 17 out of 20 experiments. Since the opening and closing of ion channels is believed to be random, a large number of experiments were performed in order to ensure that the effect observed was not the result of random fluctuations in channel activity. Control experiments in which saline was microperfused onto the C1 neurone showed no such reduction in channel activity. A reversal of the effect of 5-HT was found in only 5 experiments. In the example shown in figure 3.9 a reduction in the channel amplitude was observed on reversal of the 5-HT effect. Such a reduction in channel amplitude was observed in some other records. The cause of this amplitude reduction may be a hyperpolarising shift in Vm, since no attempt to voltage clamp the cell during these experiments was made. The failure to obtain a reversal of the 5-HT effect in many cases was probably due to a very slow diffusion of 5-HT from the site of the C1 neurone. The response to 5-HT in a voltage-clamped C1 neurone did not desensitise, and in the continued presence of 5-HT a maintained inward current was observed.

It was more difficult to asses the effect of 5-HT on the smaller unitary outward currents, since the activity of the larger channels often obscured the smaller unitary currents. It did, however, appear that the activity of the small channels was largely unaffected by application of 5-HT to the C1 neurone. Figure 3.10 shows a record in

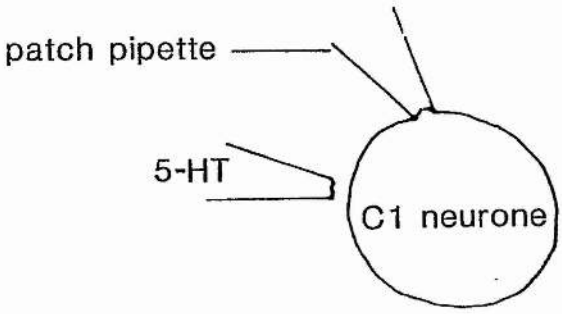
Figure 3.9

The effect of 5-HT on the large K channels recorded in a cell-attached patch of the C1 neurone. Upper trace, Single channel currents recorded under control conditions. Middle trace, Single channel currents recorded after 1.5min application of 5-HT by microperfusion. 5-HT caused a reduction in the total open time of these channels from 9.8 to 0.4%. Lower trace, Reversal of the 5-HT effect after removal of the microperfusion pipette and allowing the 5-HT to diffuse away for about 1min. A small reduction in the channel amplitude was observed on recovery. Vp= +69mV. Records were filtered at 1KHz.



control

5-HT



recovery

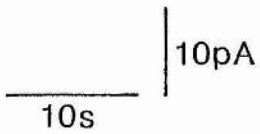
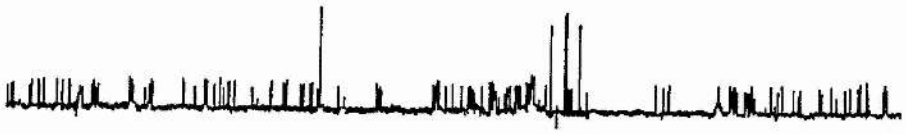


Figure 3.10

Smaller outward channel currents present during application of 10^{-4}M 5-HT. Top trace - recording from a cell-attached patch in control conditions. Bottom trace - recording after 1min application of 5-HT by microperfusion, showing a dramatic reduction in the activity of the larger channels during the continued presence of the small channels. Vp=0mV. In the upper trace some inward going events of varying amplitudes are evident. These events appeared to be due to break-down of the membrane seal, since they were of variable amplitude.

control



5-HT

2pA

4s

which 5-HT dramatically reduced the open time of the 54pS K channel, but appeared to have little effect on the open time of the 14pS channel. However the activity of this smaller channel, before the application of 5-HT cannot be accurately assessed due to the presence of the 54pS channel.

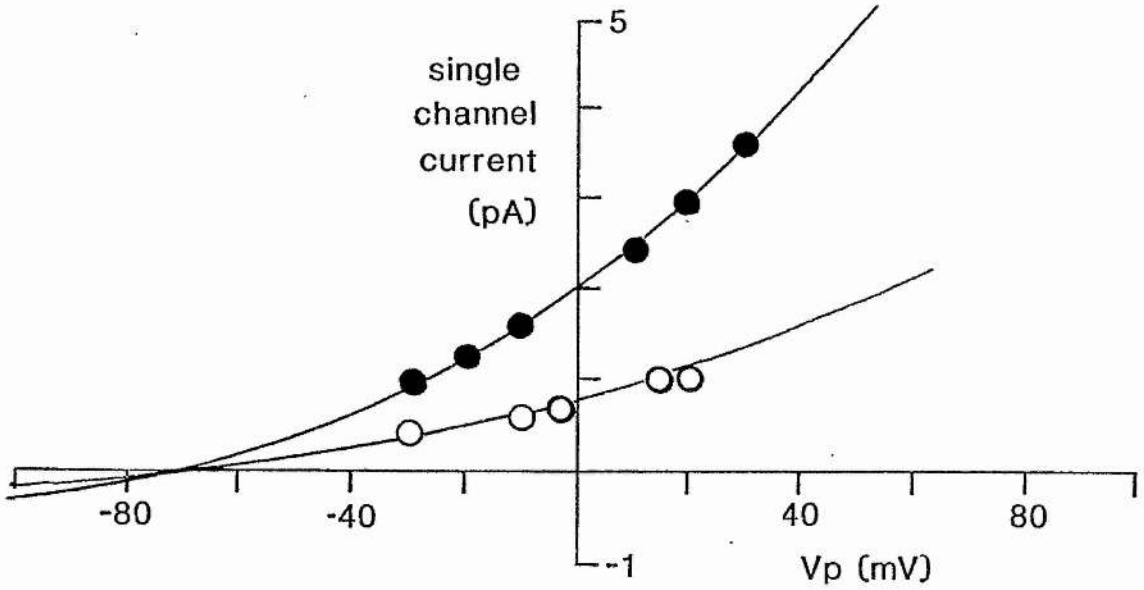
3.6 EFFECT OF 5-HT IN THE PRESENCE OF Co

The response to 5-HT in a voltage-clamped C1 neurone has been shown to be blocked by 1mM Co^{2+} ions (Barnes, Cottrell and Dunbar, in preparation). This suggested that the K channels, whose activity is reduced by 5-HT, may be Ca-dependent. However, a similar voltage-dependent 5-HT response, studied in Helix neurones by Deterre et al (1982), was also blocked by Co^{2+} ions, but was reported to be independent of intracellular Ca concentration. Experiments were performed here to determine the mechanism by which Co blocks the 5-HT response. Two experimental regimes were used, one in which Co was present in the bath and pipette solutions, and one in which 1mM Co was present in the patch pipette alone.

In the presence of 1mM Co in both the bath and patch pipette, recordings of the two commonly observed unitary currents could be made. Figure 3.11 shows the I-V relationships of unitary outward currents recorded from a cell-attached patch in the presence of Co. These relationships are similar to those obtained from unitary outward currents recorded in control conditions. Both sizes of unitary currents recorded in the presence of Co could be fitted with the theoretical relationship derived from the GHK equation for a K

Figure 3.11

The I-V relationship of outward channel currents of two amplitudes recorded with 1mM Co in both bath and pipette solutions. Both amplitudes of single channel currents could be fitted with the theoretical relationship of the GHK equation for a K current. Permeability values calculated from the data points assuming an intracellular K concentration of 98mM and a resting membrane potential of -60mV, were, $2.3 \times 10^{-13} cm^3.s^{-1}$ for the larger channels (filled circles) and $8.9 \times 10^{-14} cm^3.s^{-1}$ for the smaller channels (open circles). The slope conductances of these channels at Vp= +10mV were 50pS and 18pS respectively.


single
channel
current
(pA)
5
-1
-80
-40
40
80
Vp (mV)

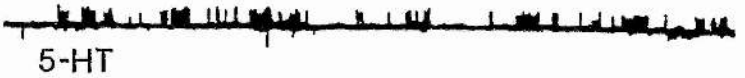
current. The large channels had a P_K value of 2.3×10^{-13} cm.^{3}s^{-1} and a conductance at +10mV of 50pS. The smaller channels had a P_K value of 8.9×10^{-14} cm.^{3}s^{-1} and a conductance at +10mV of 18pS. These values agreed reasonably closely with the values obtained under normal conditions, suggesting that the channels observed were the same. The activity of these channels also appeared similar to channels recorded under normal conditions. However, since no control could be obtained from the same patch in the absence of Co, an effect of Co^{2+} ions on the kinetic parameters of the channels could not be ruled out.

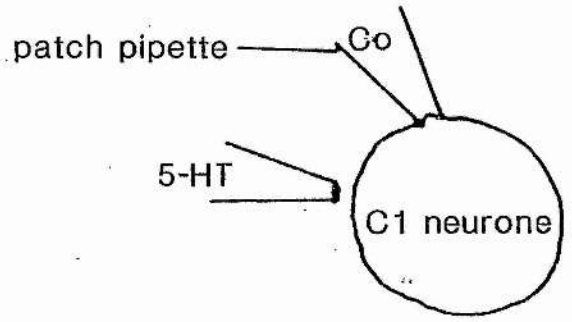
With 1mM Co present in the patch pipette alone, 5-HT was still effective in reducing the percentage open time of the 54pS K channels (see figure 3.12). This suggested that the site of action of Co^{2+} ions in blocking the effect of 5-HT was not at the level of the K channel itself. However, when 1mM Co was present in both the bath and patch pipette, the activity of the 54pS K channel could not be reduced by application of 5-HT to the C1 neurone (see figure 3.13). This blockade of the 5-HT response, seemingly without effecting the K channels involved, argued against the action of Co being an indirect block of Ca-dependent K channels. The action of Co on the response could be unrelated to its blocking action on Ca channels. For example Co^{2+} ions may interact with the 5-HT receptor to prevent the binding of 5-HT to the receptor. The effect of Co^{2+} ions could also suggest a requirement for Ca^{2+} ions in the production or action of the intracellular messenger. In studies on a similar response, Deterre et al (1982) found that Co^{2+} ions blocked a cAMP-mediated 5-HT response, as well as the response to intracellular injection of cAMP. However, these complicated actions of Co^{2+} ions do not rule out an effect of 5-HT on a

Figure 3.12

The effect of 5-HT on single channel K currents in the presence of 1mM Co in the patch pipette. The upper trace shows the control recording in the absence of 5-HT. The middle trace shows the recording 1min after application of 10^{-4}M 5-HT by microperfusion. This resulted in a reduction of of the total open time of the large K channels. The lower trace is a section of recording, showing some recovery of channel activity 3.6min after removal of the microperfusion pipette and allowing 5-HT to diffuse away. Vp= +2mV. Records were filtered at 2.5KHz.


control


5-HT


patch pipette
Co
5-HT
C1 neurone


recovery

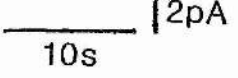
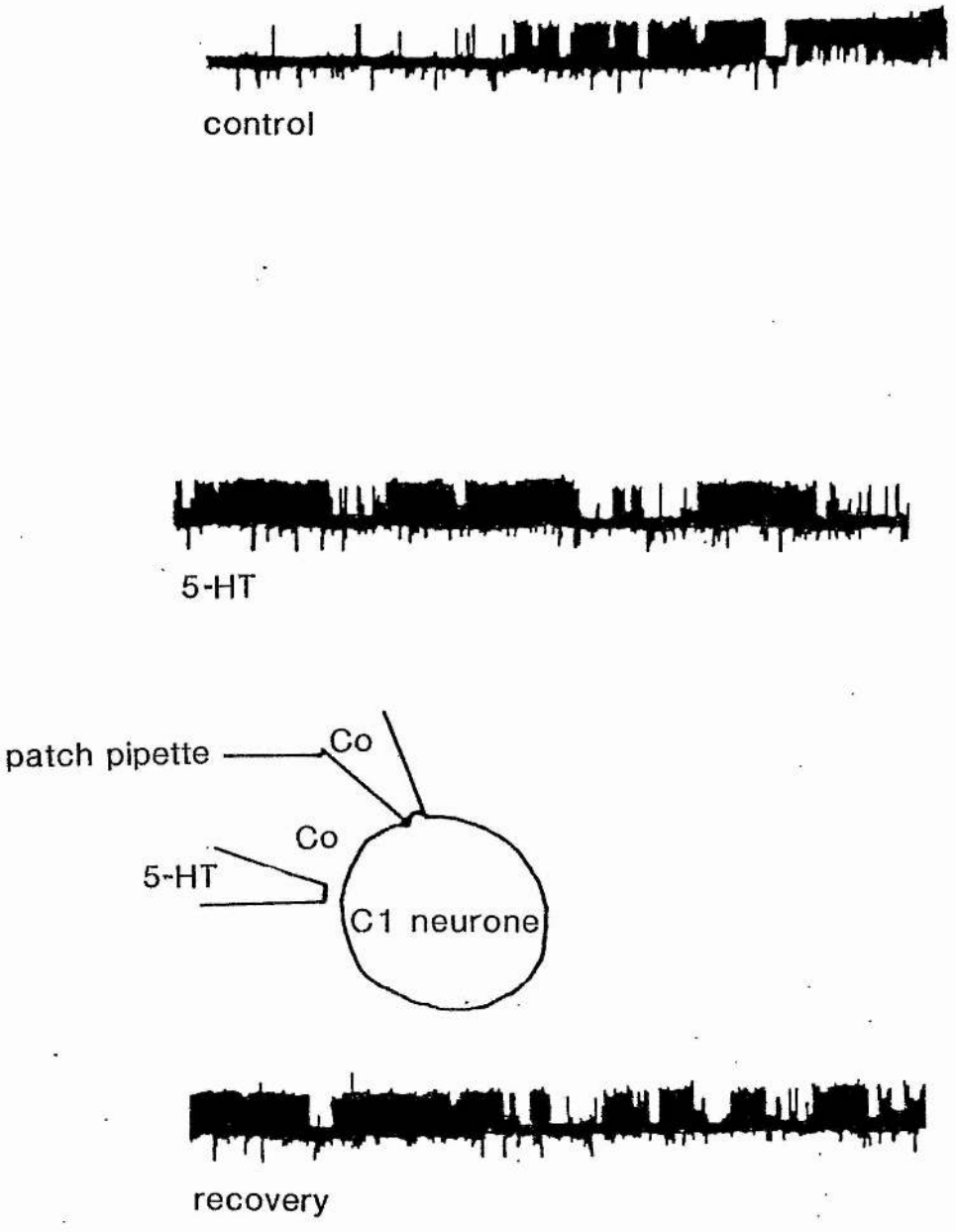

2pA
10s

Figure 3.13

The effect of 5-HT on the large K channels in the presence of 1mM Co in both the bath and patch pipette. The upper trace shows a section of recording under control conditions. The middle trace shows the recording 0.6min after beginning application of 5-HT by microperfusion. No effect of 5-HT was seen on these K channels under these conditions. In the lower trace a section of recording after a further 2min of 5-HT application is shown. Vp= +25mV. Records were filtered at 2.5KHz.


control
5-HT
patch pipette
Co
Co
5-HT
C1 neurone
recovery
4pA
10s

Ca-dependent K current. The Ca-dependency of K channels recorded in the C1 neurone was examined directly by perfusing isolated inside-out patches of membrane with different Ca concentrations.

3.7 EXAMINING THE Ca-DEPENDENCY OF THE K CHANNELS

Isolated inside-out patches were perfused on the inner surface by transferring the pipette, and thus the patch of membrane, to the microperfusion chamber described earlier. Four solutions of different free Ca concentrations were perfused over the patch of membrane. The composition of these solutions is detailed in table 2.1 (solutions 4 to 7). All of the solutions contained 5mM EGTA to chelate Ca^{2+} ions. Different quantities of $CaCl_2$ were added to give the desired free Ca concentration. The free Ca concentrations were calculated using a program written for the BBC microcomputer (Nicol, 1985). This program took into account the other ions and ligands present, as well as the temperature, which was given as 22°C. The free Ca^{2+} ion concentrations are listed in column 11 of table 2.1. In these experiments, the solution present in the bath contained no added Ca (solution 1, table 2.1). This minimised contamination of Ca from the bath into the microperfusion chamber. This solution was also used in the patch pipette. The solutions perfusing the internal surface of the patch of membrane contained 100mM K^+ ions, thus roughly mimicking the K gradient across the cell-attached patch. This physiological K gradient enabled a comparison of the single channel conductance of unitary currents recorded in isolated and cell-attached patches.

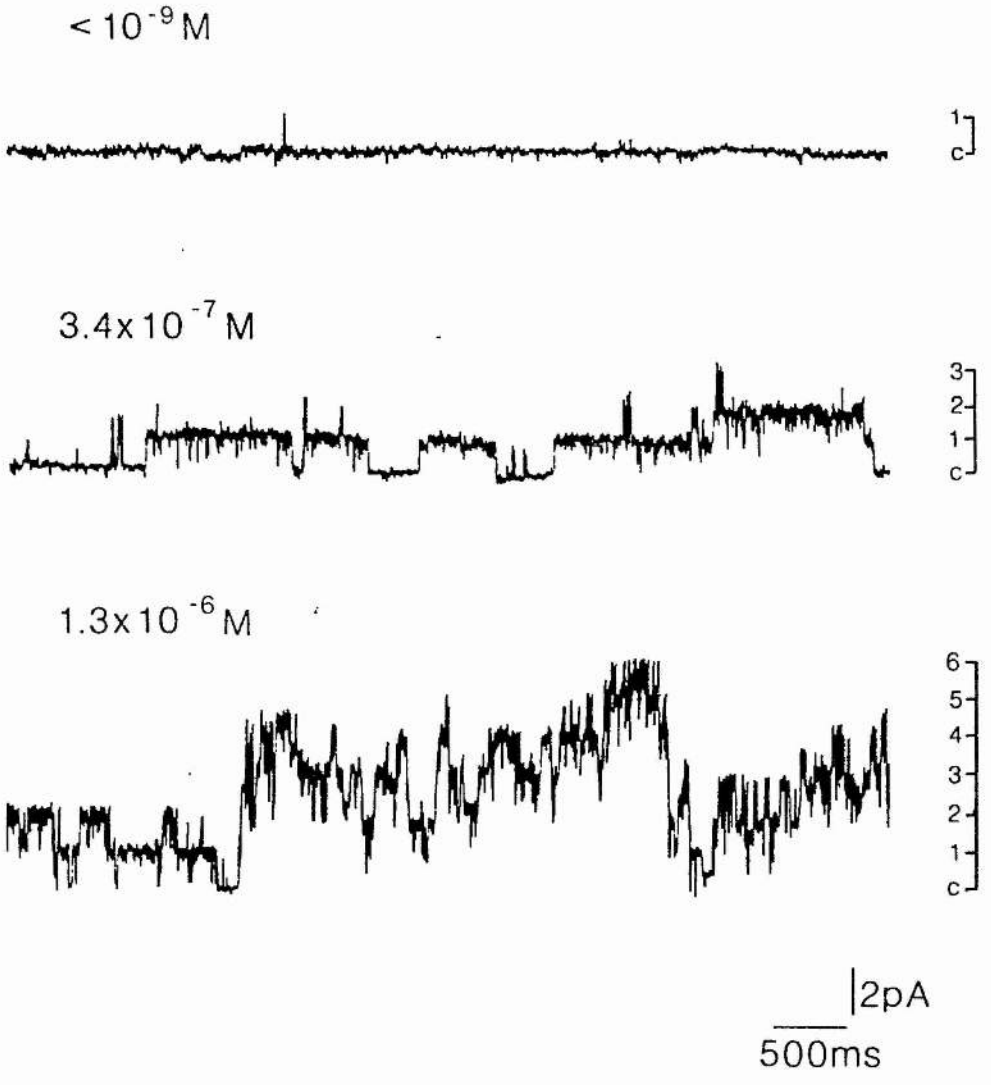
3.7.1 Ca-DEPENDENT K CHANNELS

Some channels observed in isolated inside-out membrane patches were found to be activated by an increase in the Ca^{2+} ion concentration on the inner surface of the membrane. Figure 3.14 shows an example of recordings from one patch of membrane, in which the activity of the channels increased with increasing Ca^{2+} ion concentration. In a solution containing less than 10^{-9}M free Ca, only an occasional channel opening could be seen in this record. When the solution perfusing the patch was changed to one containing $3.4x10^{-7}$M free Ca, more frequent channel openings to two superimposing levels were observed. During perfusion of the patch with $1.3x10^{-6}$M free Ca solution the simultaneous opening of six channels of the same amplitude could be observed, suggesting the presence of at least six channels in the patch of membrane. Increasing the Ca concentration further to $2.6x10^{-5}$M caused a further small increase in channel activity, but did not increase the number of simultaneously open channels. These Ca-dependent channels generally occurred in large numbers (4 to 8 per patch). Records containing only one Ca-dependent K channel in an isolated inside-out membrane patch were never observed.

The large number of Ca-dependent K channels present in each patch precluded a detailed kinetic analysis of the open and closed times. It was, however, possible to use a program for the BBC microcomputer to measure the total channel open time for each level. This value was then used to calculate the mean percentage open time per channel, assuming that the number of channels present in the patch was

Figure 3.14

K channels activated by increasing the Ca concentration on the inner surface of an isolated inside-out patch of membrane. Records were filtered at 2.5KHz. Vp= 0mV. Upper trace, Section of recording in a solution containing $<10^{-9}$M free Ca^{2+} ions. Only one isolated opening can be seen in this record. Middle trace, Section of recording from the same patch, in a solution containing $3.4x10^{-7}$M free Ca^{2+} ions. Openings to 3 levels can be seen in this record. Lower trace, section of recording from the same patch, in $1.3x10^{-6}$M free Ca solution. Openings to six levels can be seen in this record, indicating the presence of at least six channels of the same amplitude in this patch of membrane.


< 10^-9 M
1
c
3.4x10^-7 M
3
2
1
c
1.3x10^-6 M
6
5
4
3
2
1
c
2pA
500ms

equivalent to the maximum number of simultaneously open channels. A plot of the percentage open time against pCa is shown in figure 3.15. This relationship is probably sigmoidal as observed by Barrett, Magleby and Pallotta (1982) for the Ca-dependent K channels of rat myotubes. This sigmoidal relationship is not evident in the results presented here, since solutions of the pCa range 8 to 7 were not used. In later experiments, attempts were made to obtain a more complete plot of percentage open time/pCa by including solutions in this range. However, in these experiments no Ca-dependent K channels were observed. This lack of Ca-dependent K channels may have been due to a seasonal variation (see discussion). Ca-dependent K channels were seen in only 7 out of 33 isolated inside-out membrane patches.

I-V relationships of Ca-dependent K channels, recorded from isolated inside-out patches, were compared with I-V relationships of channels recorded in the cell-attached mode. An example of an I-V curve of Ca-dependent K channels recorded from an inside-out patch is shown in figure 3.16. The I-V relationship of these channels generally agreed well with the theoretical relationship calculated from the GHK equation for a K current. In the example shown in figure 3.16 the P_K value was 2.29×10^{-13} cm.^{3}s^{-1} and the conductance at a patch potential of +10mV was 55pS. The conductance of Ca-dependent K channels in isolated patches was very variable. The mean conductance at +10mV was 50 ± 11pS (n=6). Since the concentration of K^+ ions and Vp can be more accurately controlled in these experiments than in cell-attached patch experiments, such an increased variability would not be expected.

Figure 3.15

Relationship between pCa (-log[Ca]) and %open time for Ca-dependent K channels. Open times were measured using a program for the BBC microcomputer, as described in the methods section. The mean percentage open time was calculated assuming that the maximum number of overlapping unitary events was equal to the number of channels present. The data plotted here are from the same patch as in Fig.3.12.

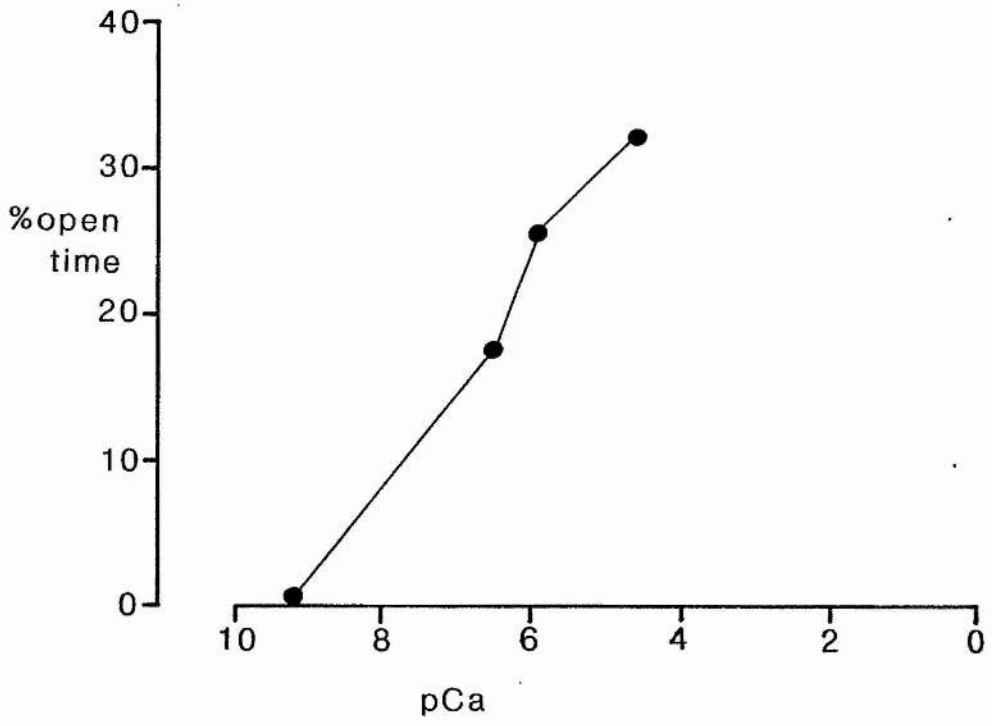
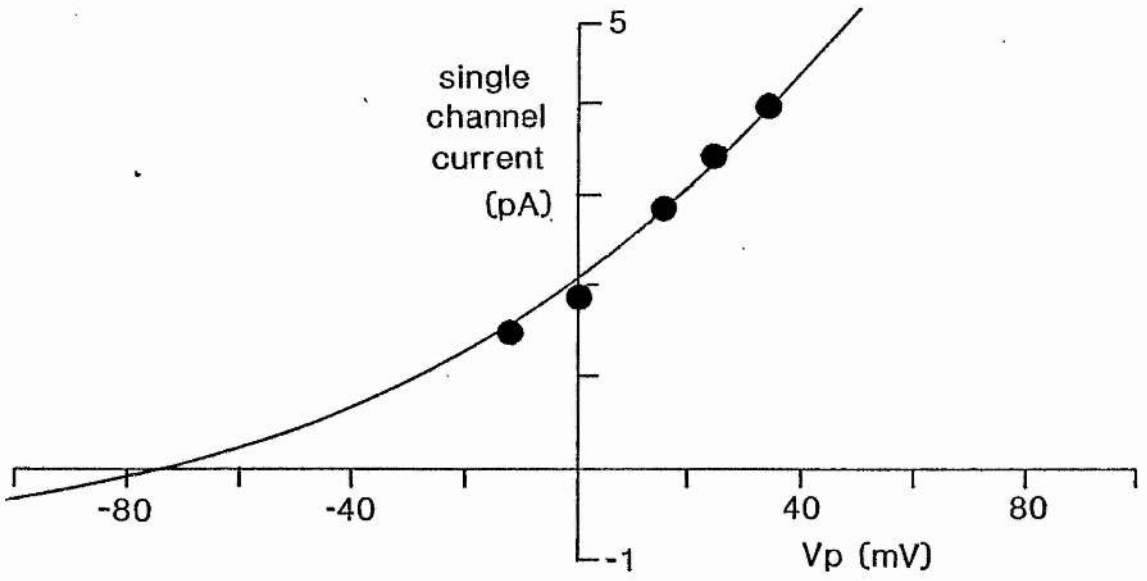

40
30
%open
time
20
10
0
10
8
6
4
2
0
pCa

Figure 3.16

I-V relationship of Ca-dependent K channels recorded from an isolated inside-out patch of membrane, with 5mM K in the patch pipette and 100mM K on the inner surface of the patch of membrane. The data have been fitted by the GHK equation for a K current . The P_K value calculated from the data points was $2.29 \times 10^{-13} cm^3.s^{-1}$. The slope conductance measured at Vp=+10mV was 55pS.


single
channel
current
(pA)
5
-1
-80
-40
40
80
Vp (mV)

3.7.2 Ca-INDEPENDENT K CHANNELS

Single channels whose activity was found to be independent of the internal Ca concentration were also present in isolated inside-out patches of membrane. These unitary outward currents were of a similar amplitude to the Ca-dependent unitary K currents at a patch potential of 0mV. The Ca-independent channels were normally present in fewer numbers (one or two) in a patch and did not usually occur in conjunction with the Ca-dependent channels. Figure 3.17 shows a recording from an isolated inside-out patch of membrane in which the channels present were very active in a solution containing less than 10^{-9}M free Ca. The activity of these channels was not affected when the solution perfusing the patch of membrane was changed to one containing 2.6×10^{-5}M free Ca.

An example of an I-V relationship of the Ca-independent K channels is shown in figure 3.18. In general the I-V relationship of these channels could not be fitted well with the theoretical relationship calculated from the GHK equation for a K current. In figure 3.18 the solid line is the fit by eye to the data points, whilst the broken line is the fit by the GHK equation for a K current, using a mean permeability value, calculated from the data points, of 3.0×10^{-13}cm.^{3}s^{-1}. Despite the fact that the rectification of these unitary currents did not agree well with the relationship derived from the GHK equation, it is likely that these unitary currents are due to current flow through channels which are largely selective for K^+ ions. An extrapolation of the line fitted by eye would suggest a reversal

Figure 3.17

K channels whose open time was not effected by Ca concentration at the internal surface. Recordings of single channel currents from an isolated inside-out patch of membrane, perfused with solutions of different Ca concentrations. Vp= 0mV. Records were filtered at 2.5KHz. Upper trace, recording from a patch perfused with $<10^{-9}$M free Ca. Lower trace, recording from the same patch perfused with 2.6×10^{-5}M free Ca solution.

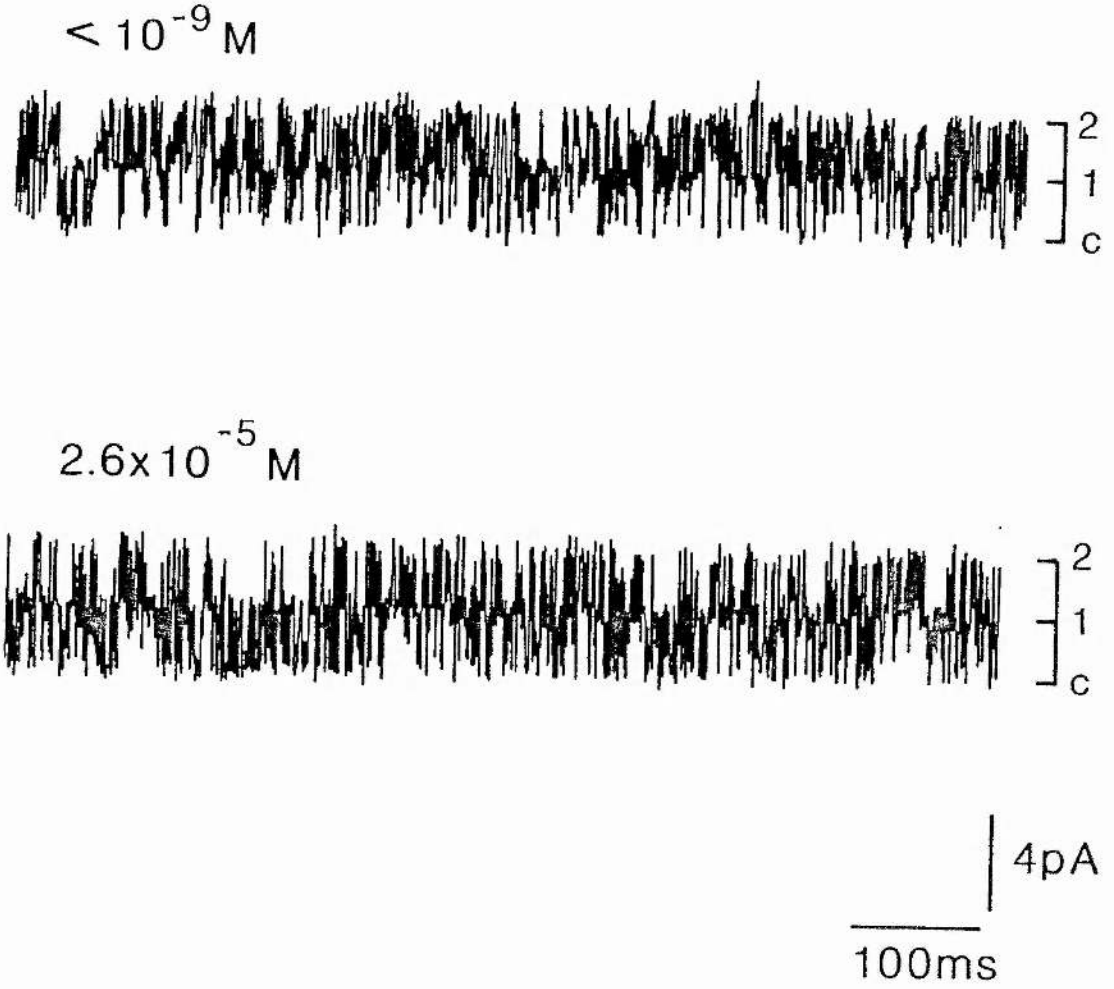
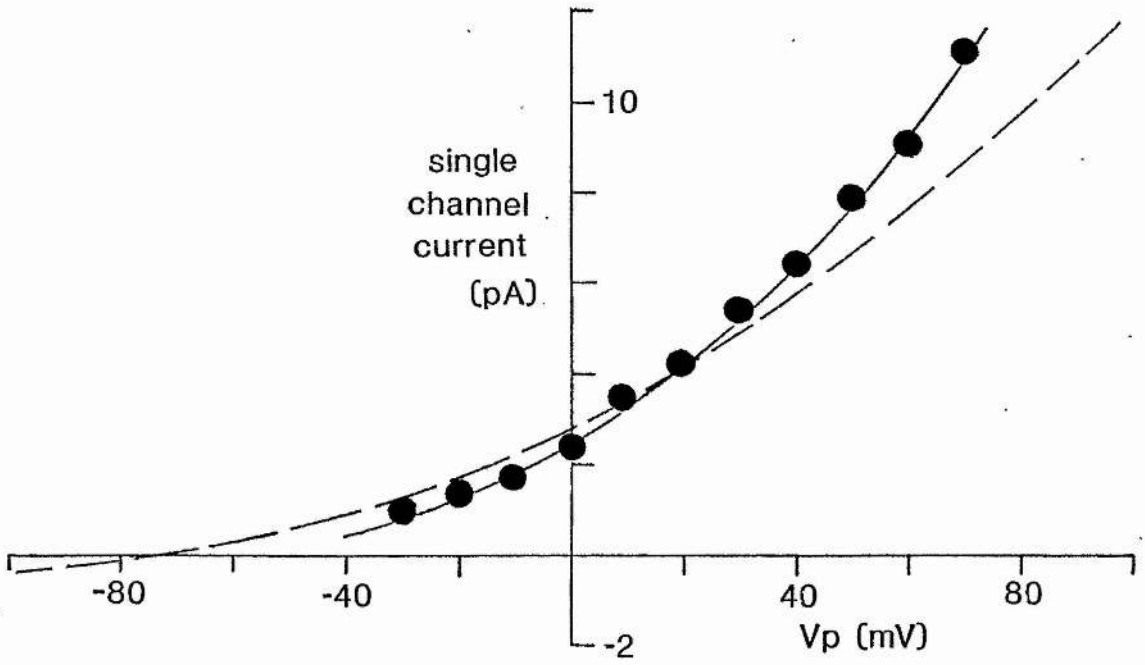

< 10^-9 M
2
1
c
2.6x10^-5 M
2
1
c
4pA
100ms

Figure 3.18

I-V relationship of Ca-independent K channels recorded from an inside-out patch with 5mM K in the patch pipette and 100mM K perfusing the inner surface of the membrane. The I-V relationship of these channels could not be fitted with the theoretical relationship calculated from the GHK equation for a K current. The broken line shows the GHK relationship, calculated using a P_K value of $3.05 \times 10^{-13} cm^3.s^{-1}$, obtained from the average P_K values of the data points. The solid line is a curve fitted by eye to the data points. The slope conductance, measured at Vp=+10mV was 90pS in this example.


10
single
channel
current
(pA)
-80
-40
40
80
-2
Vp (mV)

potential at a value more negative than -50 or -60mV. The equilibrium potentials for both Na^+ and Ca^{2+} ions were at positive potentials in this solution and the equilibrium potential for Cl^- ions was about -3mV. Therefore, the only current which could produce such an I-V relationship is one which was carried largely by K^+ ions.

The slope conductance of the unitary currents in figure 3.18 was 89pS at a Vp of +10mV. The conductance values of Ca-independent channels were variable and the mean conductance, at +10mV was 79 ± 15pS (n=9). In general the conductance values of the Ca-independent channels were higher than those of the Ca-dependent channels. However the standard deviations for both sets of data were relatively high and so a certain amount of cross-over in conductance values occurred. A Student's T-test was performed on the conductance values of the Ca-dependent and Ca-independent K channels. The results of this test suggested that, on the basis of conductance, the channels belonged to two separate populations ($p<0.01$).

3.8 EFFECT OF TEA AND Cs ON Ca-INDEPENDENT K CHANNELS

The effect of TEA and Cs on the Ca-independent channels was examined by perfusing the inner surface of isolated inside-out patches of membrane with solutions containing TEA and Cs. TEA and Cs are known to block K currents of molluscan neurones (Thompson, 1977; Hermann and Gorman, 1981; Meech and Standen, 1975; Akaike, Lee and Brown, 1978). Both TEA (20mM) and Cs (10mM) caused an apparent reduction in the single channel current when applied to the inner surface of the membrane. This effect was rapidly reversible on perfusion with the

normal intracellular solution. Figure 3.19 shows the effect of 20mM TEA on the Ca-independent K channels. The amplitude of the unitary currents appeared to be reduced from about 2.2pA to 0.8pA. This amplitude reduction is shown graphically in the form of amplitude histograms in figure 3.20. Amplitude histograms were constructed using a program written for the BBC microcomputer (see methods). Perfusing the patch of membrane with a solution containing 10mM Cs^+ ions produced an apparent reduction in the unitary currents from 2pA to 1pA (see figure 3.21). The effect of Cs^+ ions is also illustrated in the form of amplitude histograms (figure 3.22).

The apparent reduction in single channel current caused by both Cs and TEA on the internal surface was probably not a true reduction in single channel conductance, but may have been due to a very fast blocking and unblocking of the ion channels by these agents. The rapid flickering of the ion channel from the blocked to unblocked state, below the level of the resolution of the system, would be seen as a reduction in the single channel current. This mechanism of channel block by TEA and Cs has been reported in other systems (Yellen, 1984; Benham, Bolton, Lang and Takewaki, 1985).

During this type of rapid blocking action an increase in the level of noise in the open state of the channel may be observed. The amount of increased noise depends on the rate at which the channel flickers from the open to closed state in relation to the resolution of the system. If the rate of flickering is well above the resolution, no increase in the open channel noise would be detected. In the presence of TEA, a significant increase in the open channel noise was not

Figure 3.19

Effect of 20mM TEA on Ca-independent K channels. Isolated inside-out membrane patches were perfused with a solution containing 20mM TEA. Vp=0mV. Records were filtered at 2.5KHz. Upper trace, Control recording with normal 5mM K solution in the patch pipette and 100mM K solution perfusing the inner surface of the patch of membrane. Middle trace, Recording of channel activity from the same patch perfused with solution containing 20mM TEA. An apparent reduction in amplitude, from 2.4pA to 0.8pA was observed during perfusion with the TEA solution. Lower trace, Recovery of channel amplitude back to control levels after perfusion with control solution. Openings to a second level can be seen in this record, indicating the presence of at least two channels in this patch of membrane.

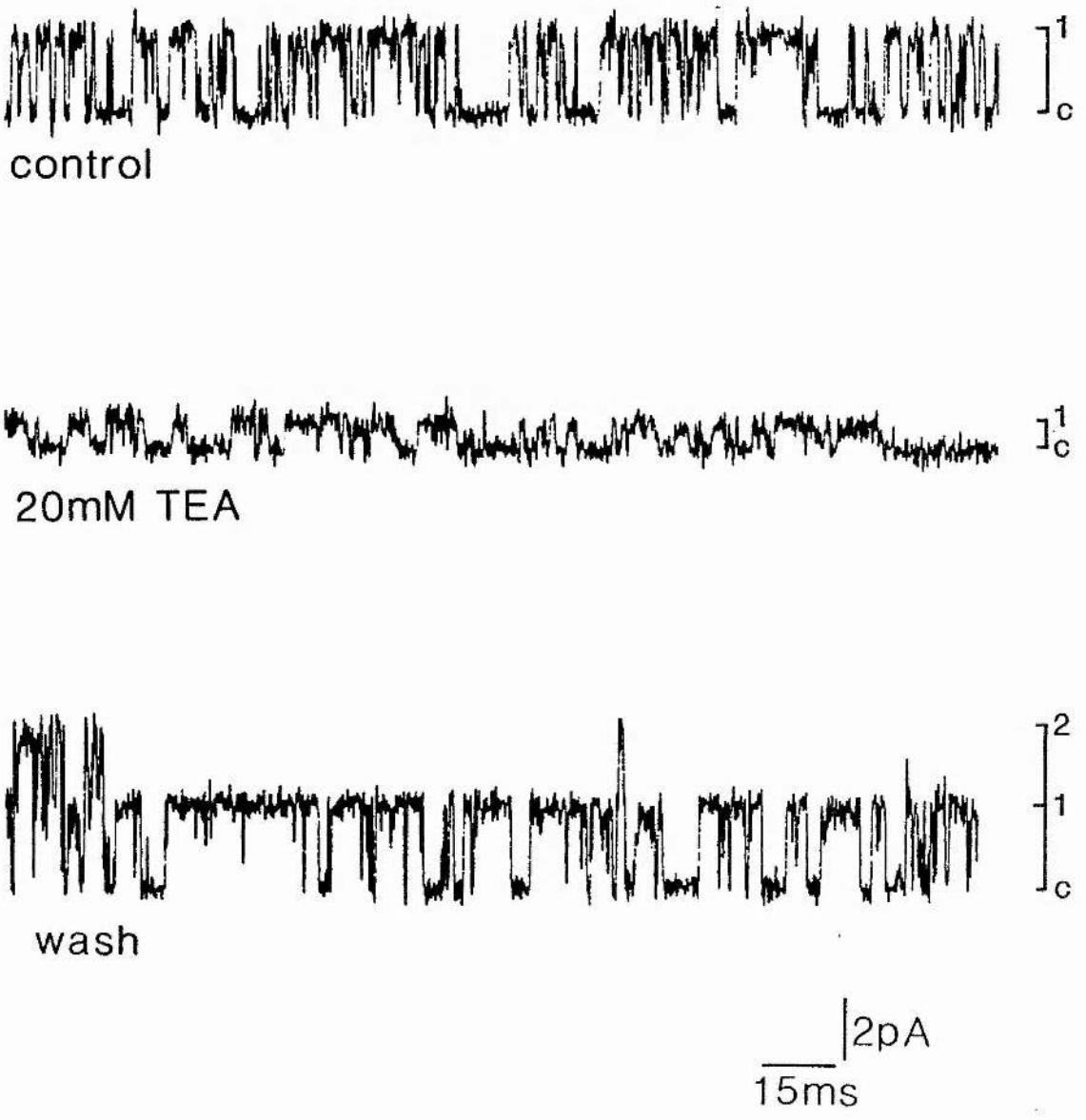
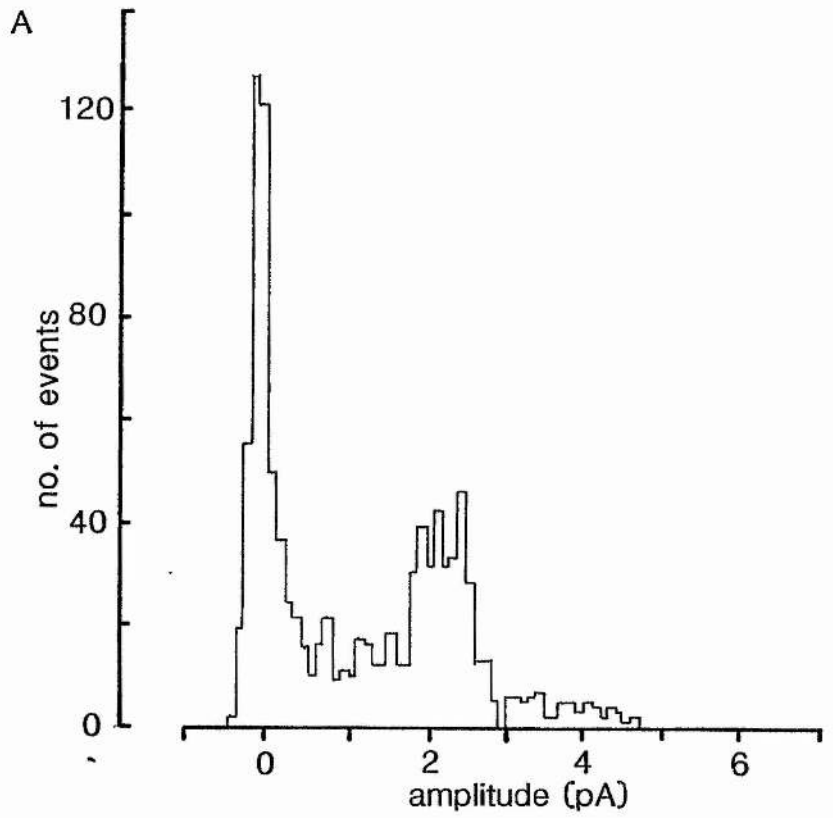

1
c
control
1
c
20mM TEA
2
1
c
wash
2pA
15ms

Figure 3.20

Amplitude histograms of Ca-independent K channels recorded from an isolated patch in control and 20mM TEA solutions. TEA caused an apparent reduction in single channel amplitude. Amplitude histograms were constructed using a program for BBC microcomputer as described in the methods section. This program measured the baseline noise in order to set the base level. A peak corresponding to baseline noise can, therefore, be seen at 0 on the abscissa. A, Amplitude histogram of channels in control conditions. A peak corresponding to one open channel level can be seen at about 2.4pA, with a second small hump at around 4pA, corresponding to two simultaneously open channels. B, Amplitude histogram of channels in the presence of 20mM TEA. A peak at about 0.8pA can be seen, due to channels of a reduced amplitude in the presence of TEA.


A
120
80
40
0
no. of events
0
2
4
6
amplitude (pA)

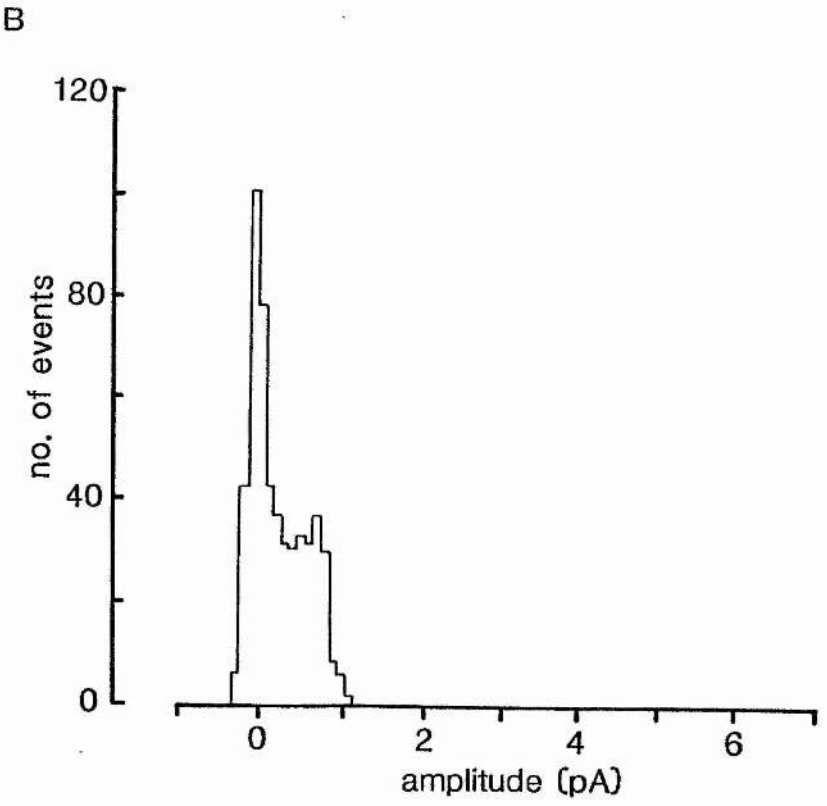

B
120
80
40
0
no. of events
0
2
4
6
amplitude (pA)

Figure 3.21

Effect of 10mM Cs on Ca-independent K channels. Isolated inside-out membrane patches were perfused with a solution containing 10mM Cs. Vp=0mV. Records were filtered at 2.5KHz. Upper trace, Control recording with normal 5mM K solution in the patch pipette and 100mM K solution perfusing the inner surface of the patch of membrane. Middle trace, Recording of channel activity from the same patch, perfused with an internal solution containing 10mM Cs. An apparent reduction in channel amplitude, from 2.2pA to 1.0pA was observed during perfusion with 10mM Cs solution Lower trace, Recovery of channel amplitude to control levels on perfusion of the patch with control solution.

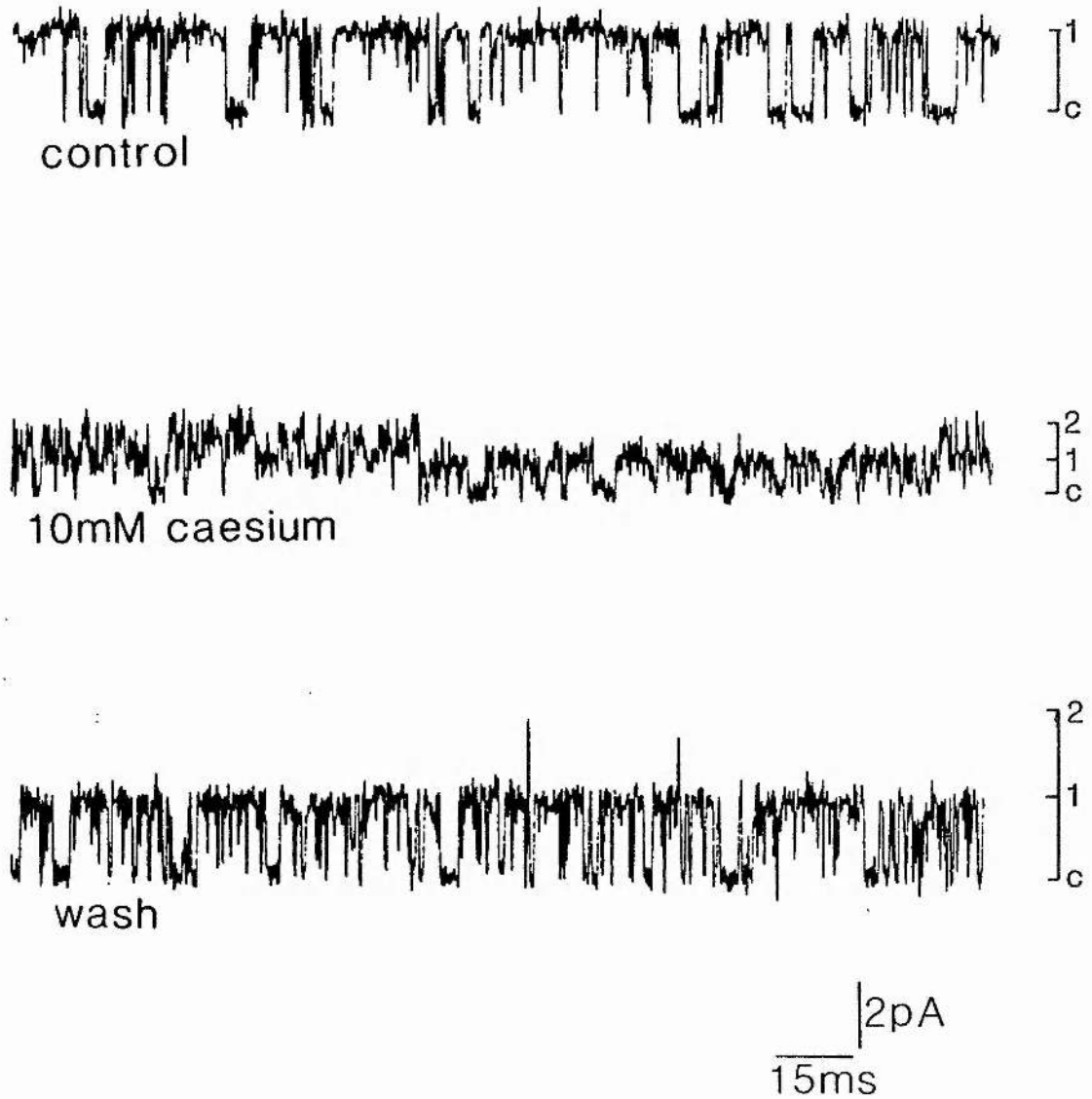
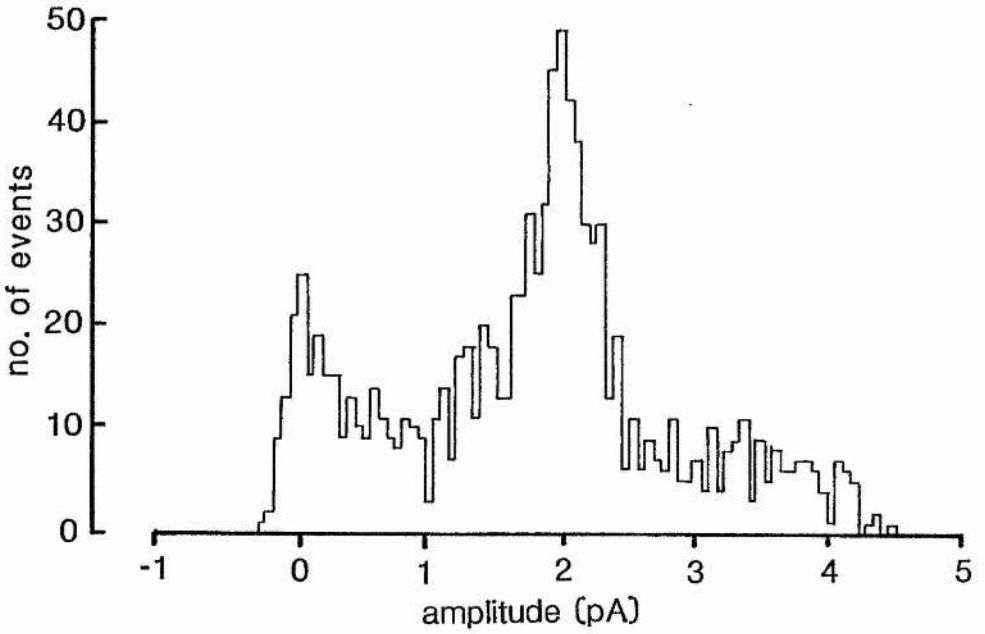

control
1
c
10mM caesium
2
1
c
wash
2
1
c
2pA
15ms

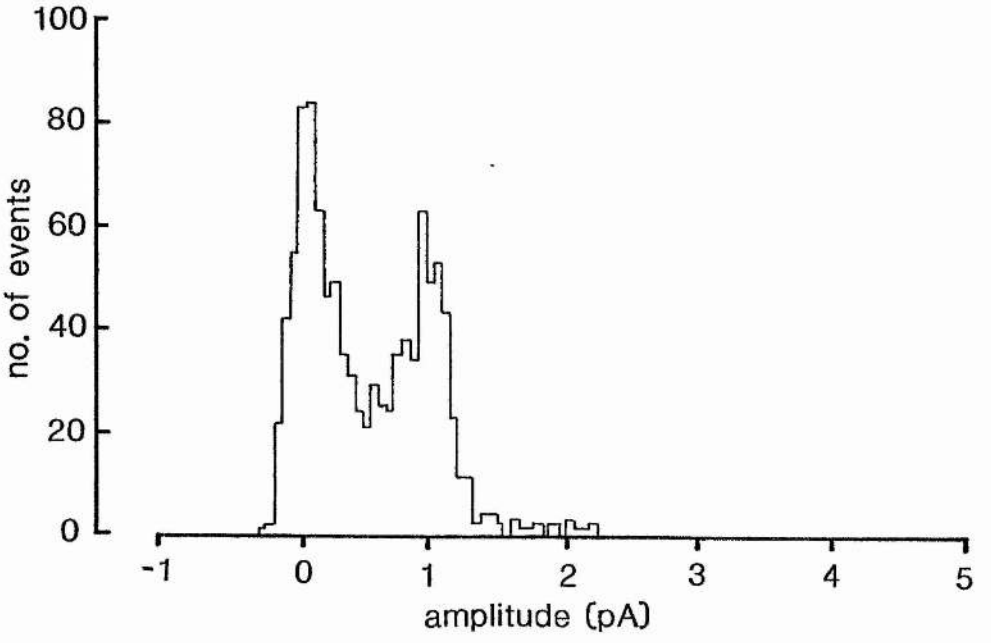
Figure 3.22

Amplitude histograms of Ca-independent K channels recorded from isolated inside-out patches in control and 10mM Cs solution to show apparent amplitude reduction with Cs. Amplitude histograms were constructed using a program for the BBC microcomputer. A peak is present at the 0 level corresponding to the baseline noise. A, amplitude histogram from control recording, showing a large peak at about 2pA and a smaller hump at about 3.5-4pA, corresponding to one and two open channels. B, amplitude histogram in the presence of 10mM Cs, showing a peak at about 1pA, corresponding to a channel of reduced amplitude.

A


50
40
30
20
10
0
no. of events
-1
0
1
2
3
4
5
amplitude (pA)

B


100
80
60
40
20
0
no. of events
-1
0
1
2
3
4
5
amplitude (pA)

observed, (see figure 3.19) suggesting that the rate of flickering was greater than the resolution of the system (2.5KHz). In the presence of Cs^+ ions a small increase in open channel noise was observed (figure 3.21). Therefore, the channel blocking by Cs^+ ions was probably slower than that by TEA. However, attempts to quantify these effects were not made.

The effect of 2mM Co^{2+} ions on the Ca-independent channels was examined by perfusing an isolated inside-out patch with a solution containing 2mM Co. No effect of 2mM Co on these channels was observed (see figure 3.23).

3.9 ANALYSIS OF OPEN AND CLOSED TIMES

Ca-independent K channels were usually present in smaller numbers than the Ca-dependent K channels. In records where only one ion channel was present, it was possible to perform a kinetic analysis of open and closed times. A comparison was made of the kinetic parameters of the 54pS K channels, recorded in cell-attached patches and the Ca-independent channels, recorded in isolated inside-out patches. A program written for the BBC microcomputer was used to measure open and closed times, which were then transferred to a Vax computer. A program using the method of maximum likelihood determined the number and means of the exponential distributions required to fit the frequency distribution histograms of open and closed times.

Figure 3.23

Lack of effect of 2mM Co on Ca-independent K channels. Upper trace, record in control conditions showing channel openings to two levels with a single channel amplitude of about 2.2pA. Lower trace, record in the presence of 2mM Co, showing no effect on channel amplitude or open time. Vp=0mV. Records were filtered at 2.5KHz.

control

2mM cobalt

2pA

15ms

This kinetic analysis was very limited, due to the small number of membrane patches which contained only a single ion channel. Table 3.1 shows a comparison of kinetic data for the 54pS K channel recorded in a cell-attached patch and the Ca-independent K channel recorded from an isolated inside-out patch. Both sets of records were obtained at a patch potential of +10mV. The open times of channels recorded from cell-attached patches could be described by the sum of two exponential distributions, whilst the closed times were described by the sum of three exponential distributions. The means of these exponential distributions and their proportions varied between patches. Examples of values from one cell-attached patch are shown in table 3.1. Although these values varied from patch to patch, the number of exponential distributions required to describe the data was consistent.

Values for both the open and closed times of the Ca-independent K channels, recorded from isolated patches, could be fitted by the sum of two exponential distributions. Examples of these values are given in table 3.1. The longer closed time, which was usually present in records from cell-attached patches was missing in records of the Ca-independent K channel, recorded in isolated patches. It was not possible to make a similar study for the Ca-dependent channels, since they were always present in large numbers in a patch of membrane.

Table 3.1

Time constants of exponential functions fitted to the open and closed time distributions of K channels, from a cell-attached patch and isolated inside-out patch, at a patch potential of +10mV. τ = exponential time constant in ms. P = proportion of channels contributing to the exponential.

	OPEN TIMES				CLOSED TIMES				
	CELL-ATTACHED			ISOLATED		CELL-ATTACHED			ISOLATED	
τ (ms)	4.8	2.1		4.8	2.4	58.22	2.17	0.55	3.10	0.46
P	0.03	0.97		0.42	0.57	0.13	0.12	0.75	0.07	0.93

The difference in the kinetics of K channels in isolated and cell-attached patches may be because they represent two different types of channel. The mean conductance value of the Ca-independent channels was higher than that of the large K channels recorded in cell-attached patches. However, Ca-independent K channels were the most frequently observed channels in isolated patches. It may be that isolation of the patch of membrane caused an alteration in the properties of the channels. The difference in the open and closed times could be explained by the loss of a regulatory substance when the patch of membrane is isolated from the cell interior.

3.10 Ca-DEPENDENCY OF THE 5-HT RESPONSE

The presence of both Ca-dependent and Ca-independent K channels of a similar amplitude in isolated patches of the C1 neurone complicated the identification of the K channels inactivated by 5-HT in cell-attached patches of the C1 neurone. To investigate further the Ca-dependency of the K current modulated by 5-HT in this cell, a series of experiments using voltage clamp techniques were undertaken. The following sections describe some experiments using both double and single electrode voltage clamp systems.

3.10.1 THE Ca-DEPENDENT COMPONENT OF THE I-V CURVE

Meech and Standen (1975) described an "N" shaped relationship between the outward current elicited by large depolarising pulses and the membrane potential to which the cell was stepped. The experiments of Meech and Standen were performed on the F1 (or A) neurone in the right parietal ganglion of Helix aspersa. The N shaped I-V relationship which they described became apparent at membrane potentials of between +50 and +130mV and was greatly reduced in nominally zero Ca solutions or in the presence of Ca channel blocking agents. They showed that this component of the I-V relationship was due to a Ca-dependent K current.

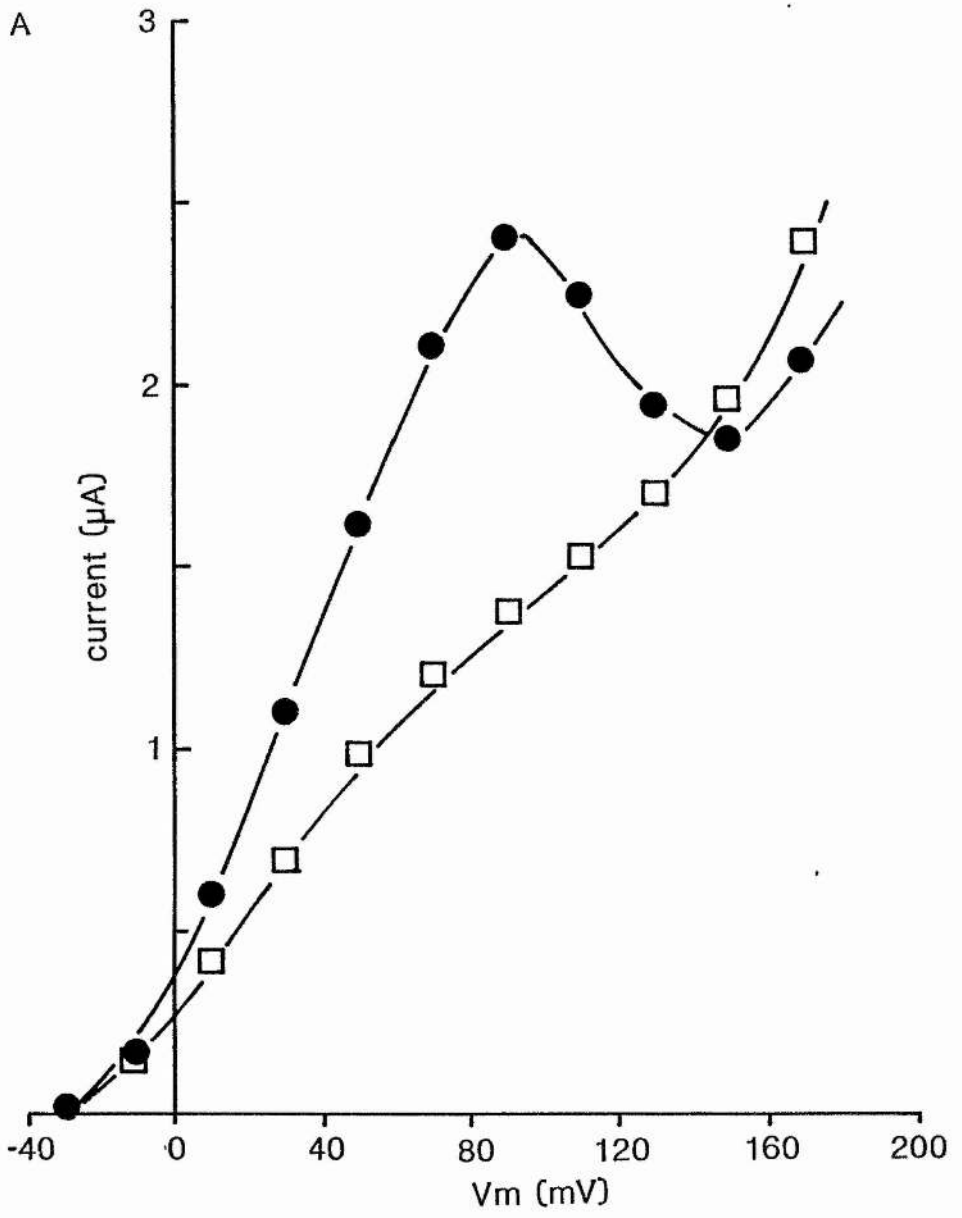
These experiments, on the F1 neurone were repeated here in order to determine that the experimental regime used was correct for recording this component of the I-V curve. A double electrode voltage clamp system was required to clamp the large neurones used in this study to very depolarised potentials. This allowed sufficient current to be passed (up to 3μA) in order to clamp the cells. This double electrode voltage clamp system also incorporated a series resistance compensation circuit to compensate for errors in the voltage record, induced by a voltage drop across series resistance components, such as the agar bridge.

It was found to be important to leave a sufficient interval between the depolarising pulses, as mentioned by Meech and Standen (1975), in order to allow the K current activation system to recover. The interval between pulses given here was 15s. Figure 3.24 shows the the I-V relationship of an F1 neurone in a solution containing 10mM Ca and in a solution containing nominally zero Ca. In control (10mM Ca) solution an N shape in the I-V curve was apparent. This component of the current was greatly diminished in a nominally zero Ca solution. Figure 3.24,B is a plot of the difference between the currents in the control and Ca free solutions, representing the component of the outward current mediated by Ca influx.

These experiments were repeated for the C1 neurone. A shallow N shape in the I-V curve of the C1 neurone was observed. However, this N shape was not as apparent as for the F1 neurone and, in general, the currents recorded from the C1 neurone were smaller than those recorded from the F1 neurone (see figure 3.25). Figure 3.25,B is a plot of the difference between the currents in Ca free and control solutions and shows a bell-shaped curve, corresponding to the component of the outward current activated by Ca influx. This component is approximately half the size of the Ca-dependent component in the F1 neurone, with a peak current of about 0.5μA at +70mV, compared to a peak Ca-dependent current in the F1 neurone of about 1μA at a membrane potential of +80mV. Current records from an F1 and C1 neurone are compared in figure 3.26. A clear "cross-over" of the current records was always observed for the F1 neurone, whilst in the C1 neurone, often only a flattening of the I-V curve was apparent. However, the

Figure 3.24

The N shaped I-V relationship of the F1 neurone and the effect of nominally zero Ca solution. Currents were elicited by 80ms depolarising pulses from a holding potential of -50mV. A, I-V relationships of an F1 neurone in control (10mM Ca) solution (●) and nominally zero Ca solution (□). A pronounced N shape in the I-V curve in control solution was apparent. This N shape was virtually abolished in nominally zero Ca solution. B, plot of the difference between currents in control and nominally zero Ca solutions, showing a bell shaped difference, similar to that seen by Meech and Standen (1975), to correspond to the Ca-dependent K current.


A
3
2
1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

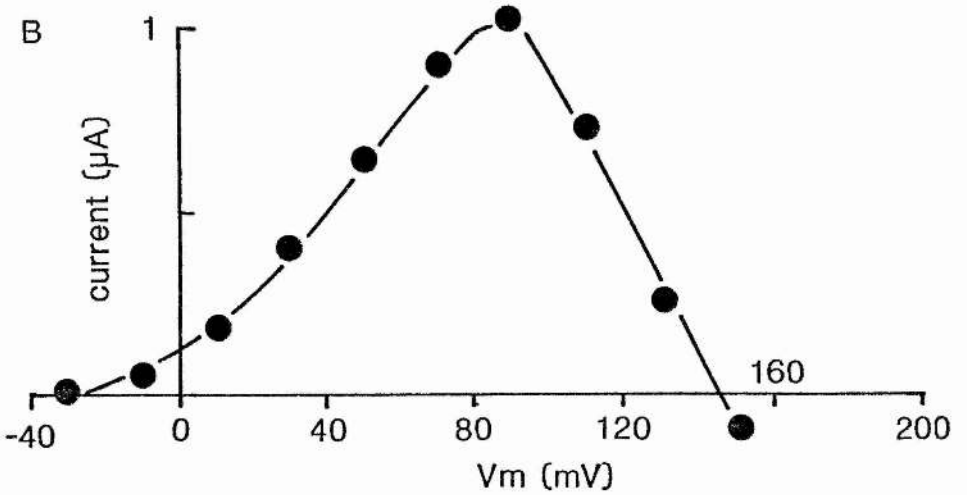

B
1
current (μA)
160
-40
0
40
80
120
200
Vm (mV)

Figure 3.25

The N shaped I-V relationship of the C1 neurone and the effect of a nominally zero Ca solution. Currents were elicited by 100ms depolarising steps from a holding potential of -50mV. A, the I-V relationship of a C1 neurone in control (10mM Ca) solution (●) and in nominally zero Ca solution (□). The I-V curve was flattened in nominally zero Ca solution and the slight N shape present in the control conditions, at about +80mV, was no longer evident. B, a graph showing the difference between currents recorded in control and nominally zero Ca solutions. The bell shaped difference was thought to correspond to the Ca-dependent K current described by Meech and Standen (1975).

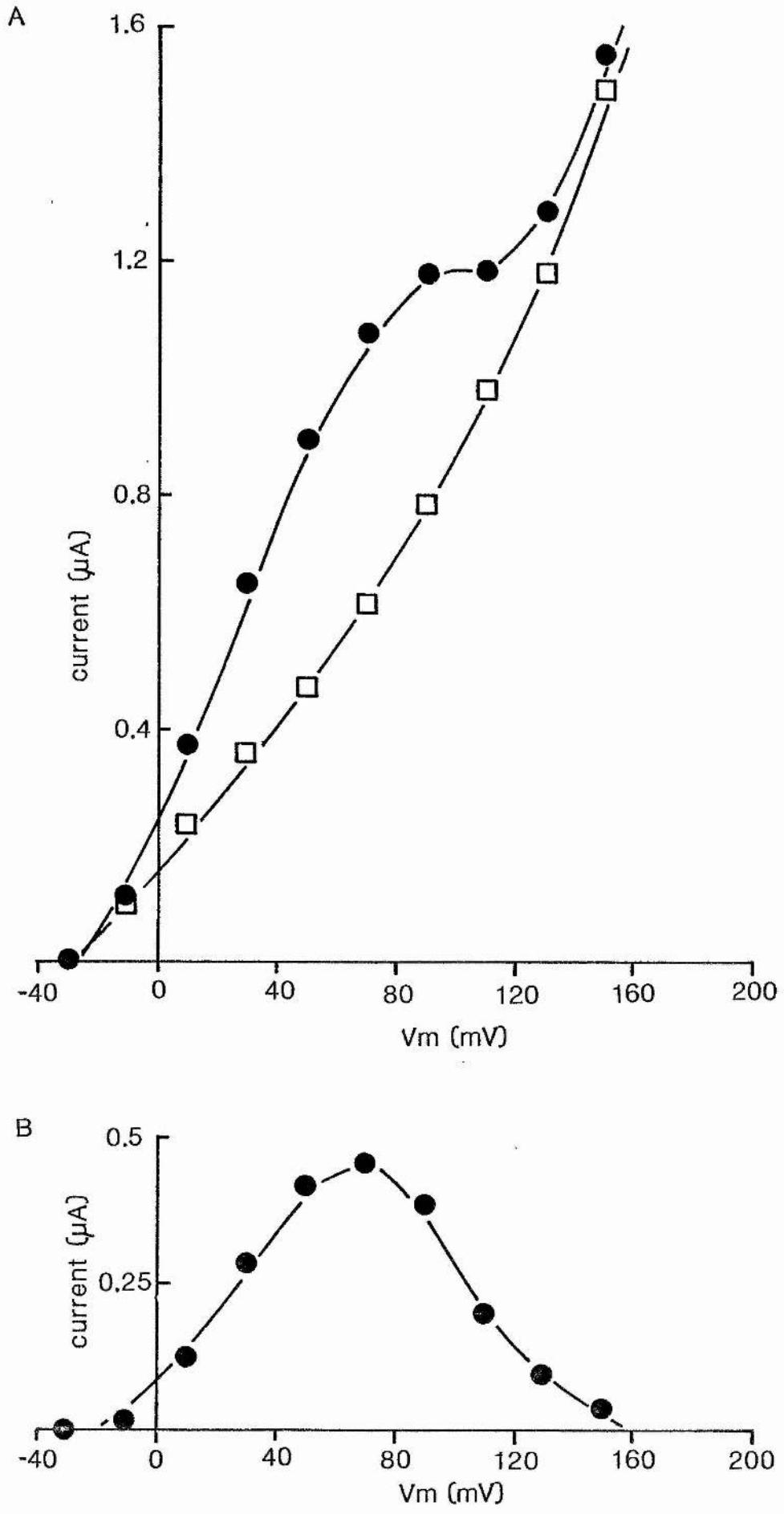
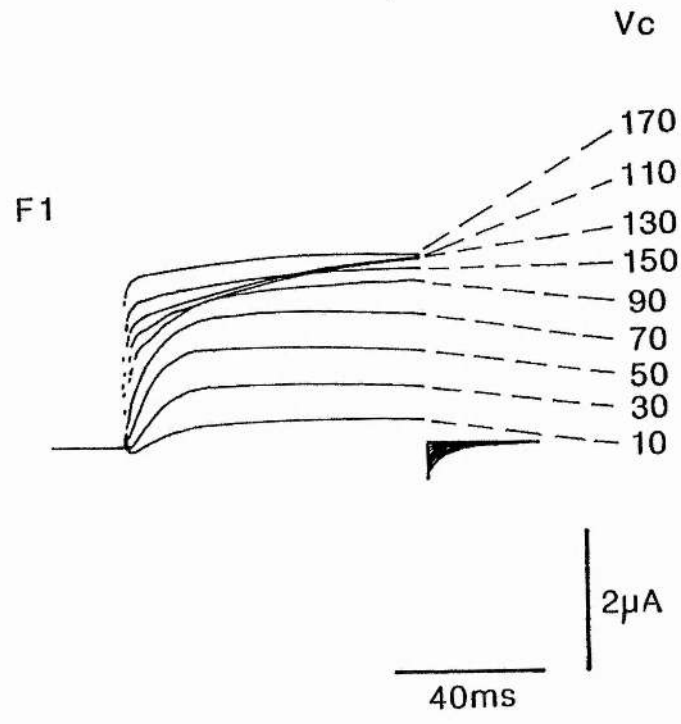
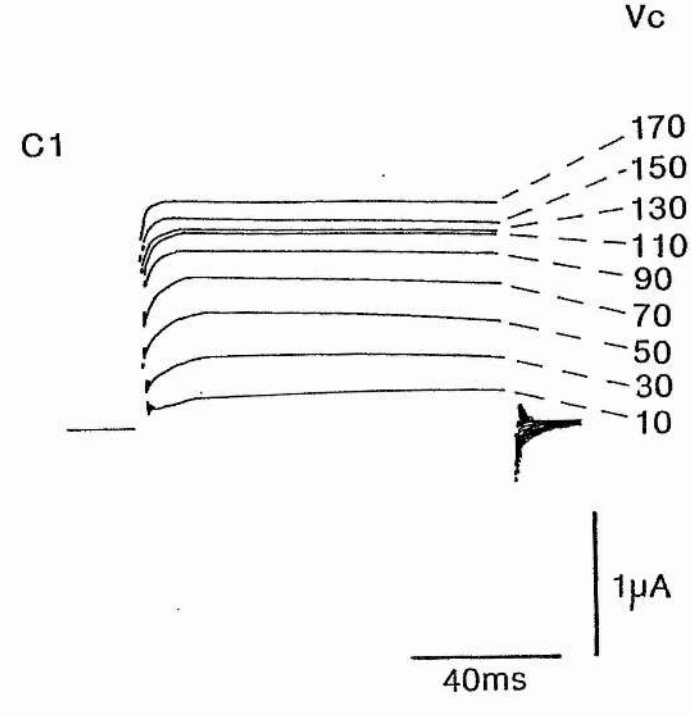

A
1.6
1.2
0.8
0.4
current (μA)
-40
0
40
80
120
160
200
Vm (mV)
B
0.5
0.25
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

Figure 3.26

Currents recorded from the F1 and C1 neurones by depolarising steps from a holding potential of -50mV. The top records show typical currents recorded from an F1 neurone. The stimulus duration in this example was 80ms. The command potentials corresponding to the currents are indicated on the right. A definite cross over of currents corresponding to the N shaped I-V relationship can be seen. The current elicited by stepping to +150mV is smaller than those elicited by steps to +110 and +130mV. The lower traces are current records from a C1 neurone, produced by 100ms depolarising steps. The currents recorded from the C1 neurone were generally smaller than those of the F1 neurone (note the different scales). In most records of the C1 neurone a definite N shape was not seen, but a flattening of the I-V curve at about +80 to +150 mV was observed, as can be seen in this example. Command potentials are indicated at the right of the currents.


Vc
F1
170
110
130
150
90
70
50
30
10
2μA
40ms


Vc
C1
170
150
130
110
90
70
50
30
10
1μA
40ms

difference between the current in control and Ca free solutions indicated that a significant Ca-dependent K current was present in the C1 neurone.

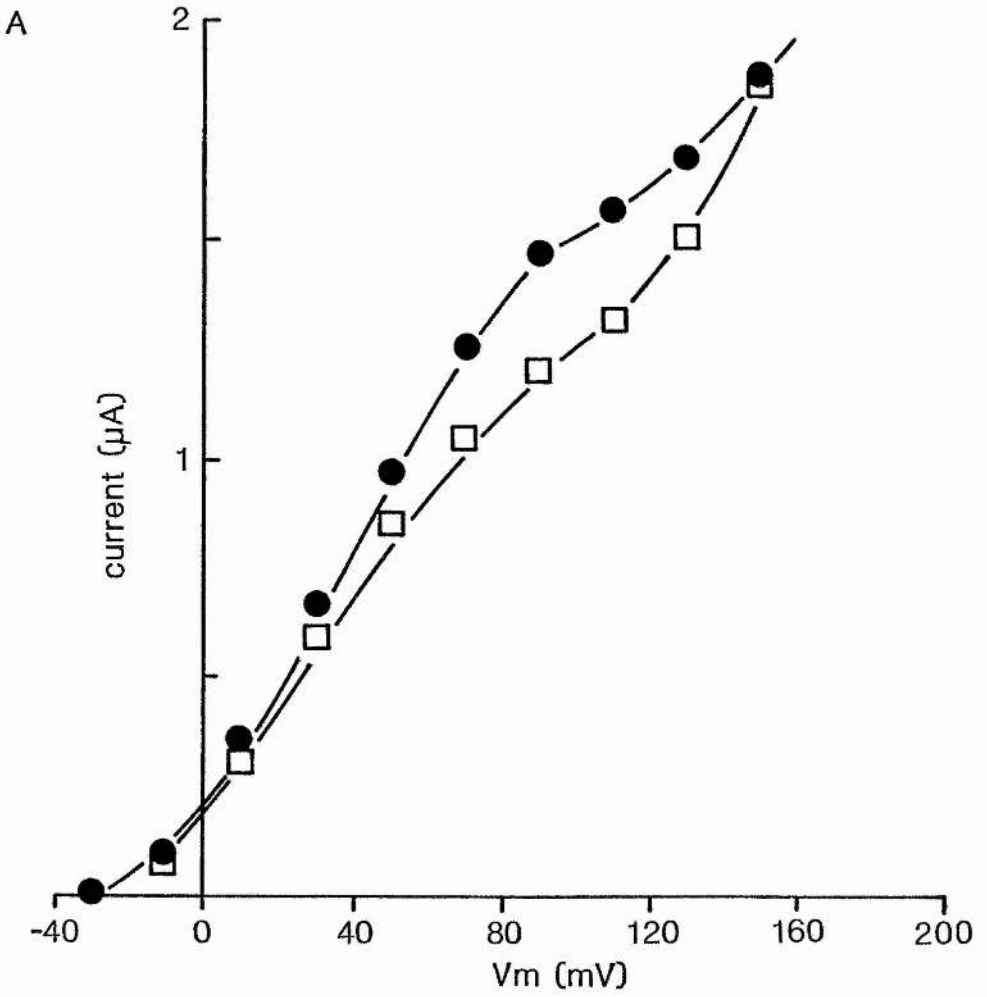
An attempt was made to increase the relative proportion of the Ca-dependent component of the I-V curve by selectively blocking the delayed rectifier current using 20mM TEA. However, at this concentration TEA markedly flattened the I-V curve, but did not enhance the N shape. It has since been shown that 7 to 10 mM TEA will enhance the N shaped component of the I-V curve (W. Lesser, personal communication).

The effect of 1mM Co on the N shaped I-V relationship of the C1 neurone was tested. Figure 3.27 shows the I-V relationships of a C1 neurone recorded in control and 1mM Co solutions. Only a very shallow N shape in the I-V curve of this C1 neurone was apparent in control conditions. This was further flattened in the presence of 1mM Co^{2+} ions, producing a roughly bell shaped difference (figure 3.27,B). This suggested that 1mM Co^{2+} ions partially blocked the component of the I-V curve which is activated by Ca influx.

5μM verapamil also appeared to have a slight affect on the Ca-dependent component of the I-V curve (figure 3.28). However, the difference in the I-V curves in verapamil and control, although showing a slight bell shape, does not exactly resemble the effect of Ca free solution. Experiments performed with 50μM verapamil indicated that, at this concentration, verapamil blocked more than just the Ca-dependent component of the outward current (see figure 3.29). A comparison of

Figure 3.27

Effect of 1mM Co on the I-V relationship of the C1 neurone. A, I-V relationships of a C1 neurone obtained by stepping Vm from a holding potential of -50mV to depolarised potentials, for a duration of 100ms, as described previously. ● - control solution. □ - 1mM Co solution. The I-V curve was flattened in the presence of 1mM Co. The difference between control and Co solutions is shown in graph B on an expanded scale. A bell shaped difference, similar to that obtained with nominally zero Ca solution was seen.


A
2
1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

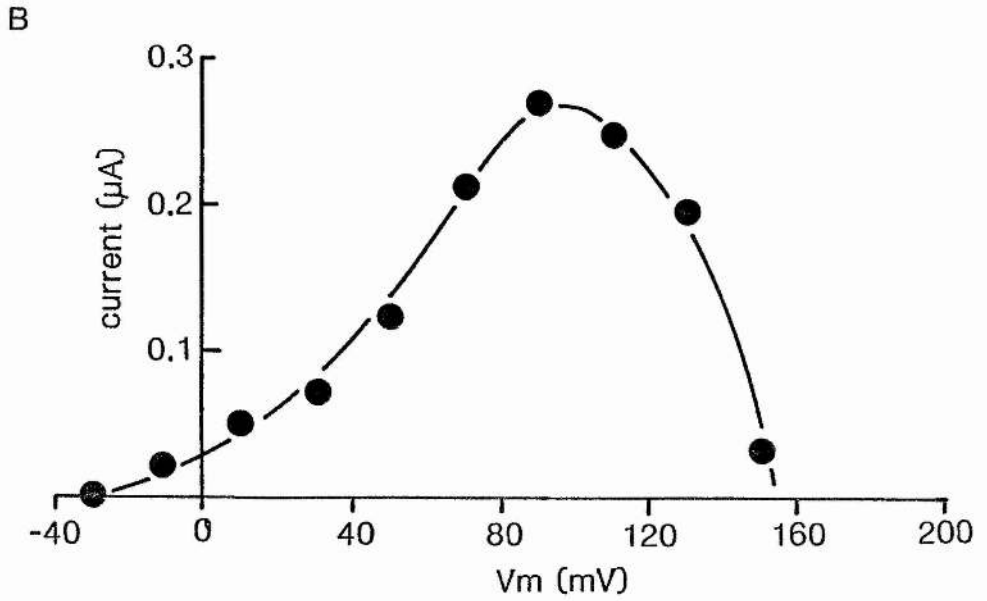

B
0.3
0.2
0.1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

Figure 3.28

Effect of 5μM verapamil on the I-V relationship of the C1 neurone. A, I-V relationships obtained by stepping Vm from a holding potential of -50mV to depolarised potentials for a duration of 100ms as described before. ● - Control. □ - 5μM verapamil. The I-V relationship was slightly flattened in the presence of 5μM verapamil. B, graph showing the difference between currents recorded in control and verapamil solutions. The slight bell shaped appearance of the difference suggests that, at least part of the outward current blocked may be the Ca-dependent K current.

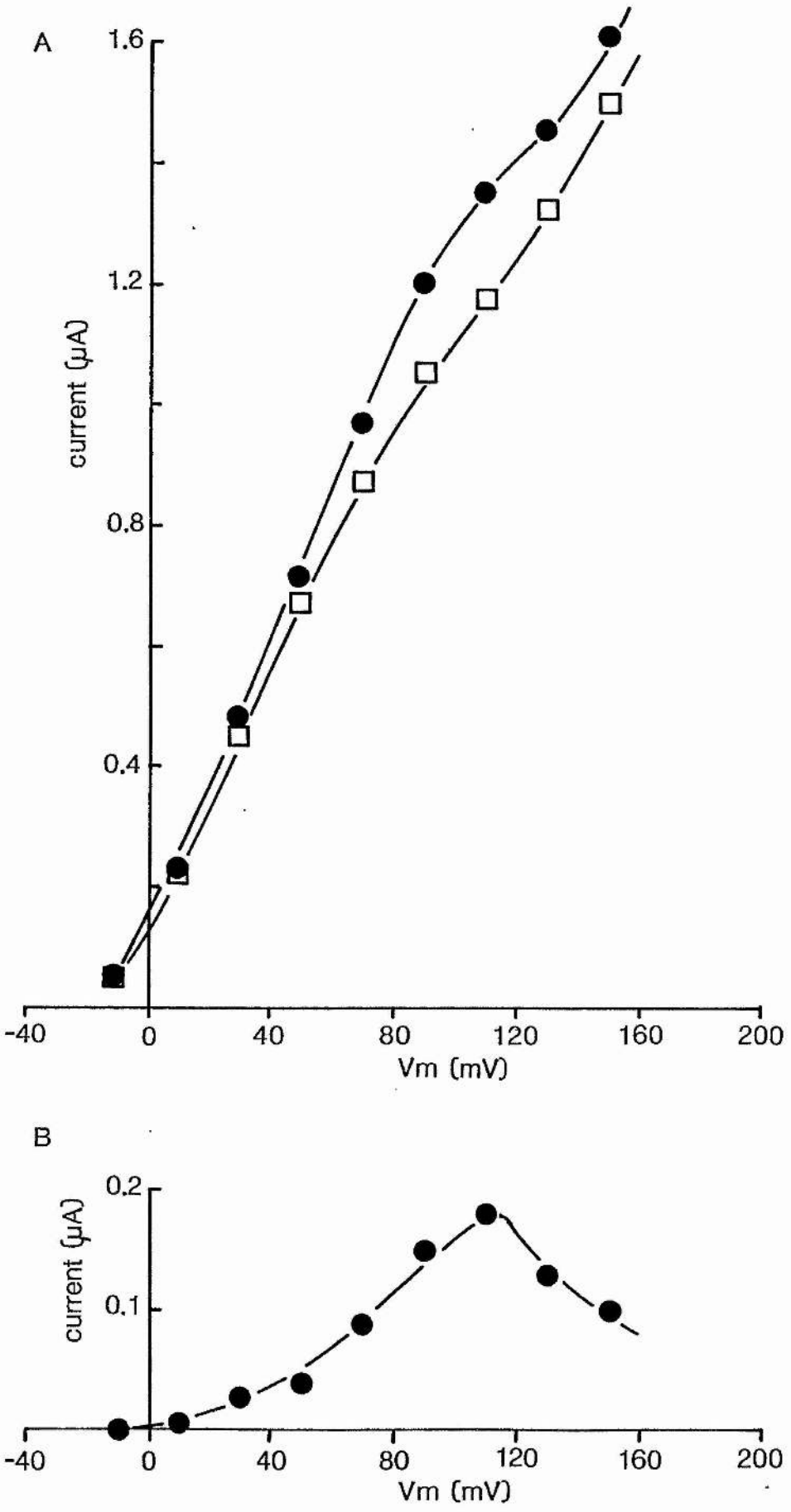
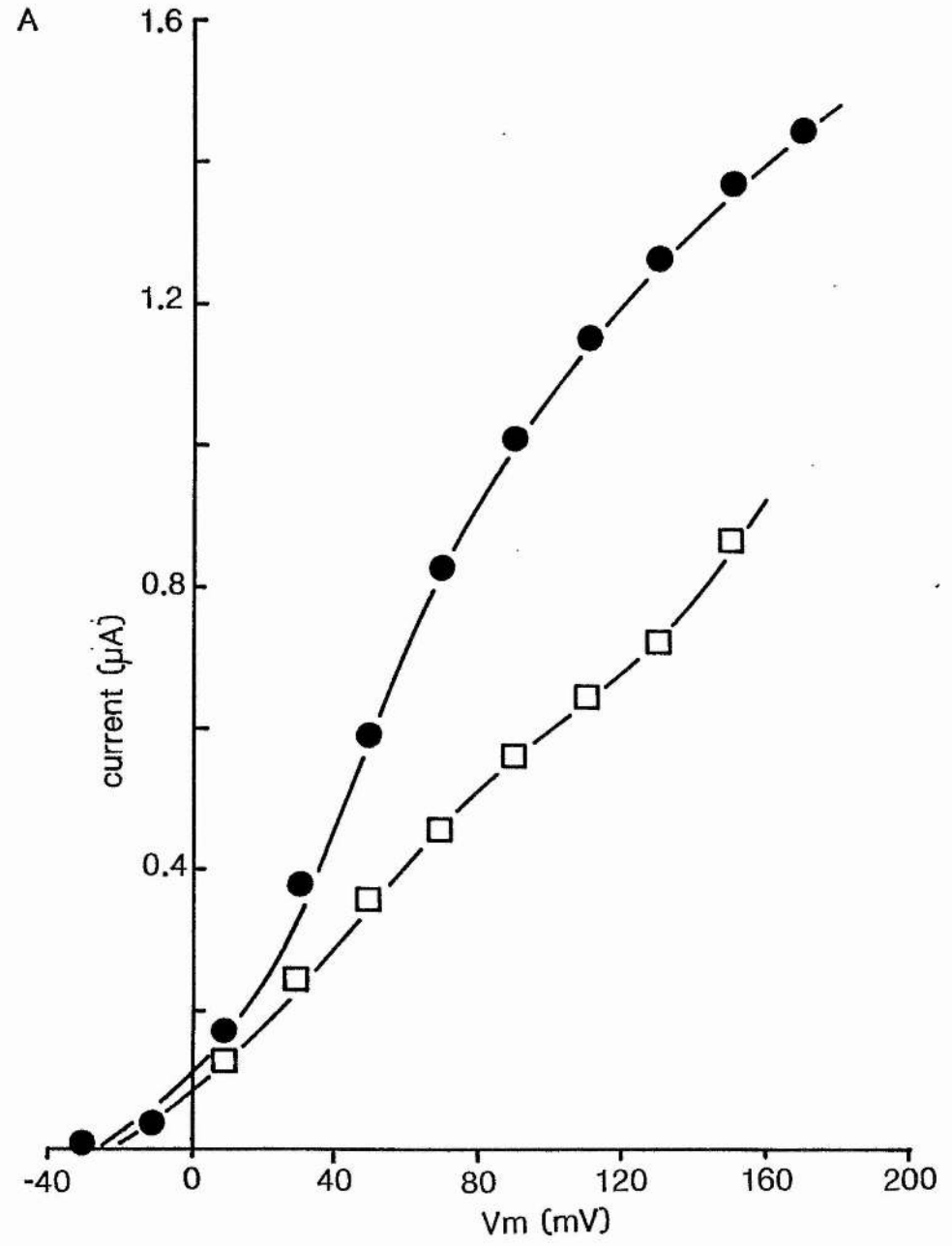
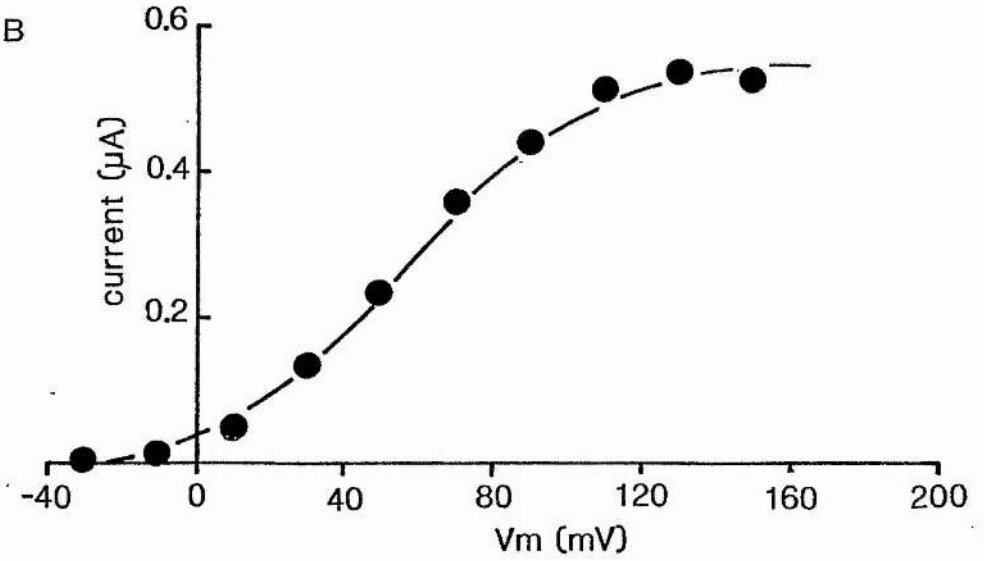

A
1.6
1.2
0.8
0.4
current (μA)
-40
0
40
80
120
160
200
Vm (mV)
B
0.2
0.1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

Figure 3.29

Effect of 50μM verapamil on the I-V relationship of the C1 neurone. A, I-V relationships of a C1 neurone obtained by stepping Vm from a holding potential of -50mV to depolarised potentials for a duration of 100ms as before. ● - control. □ - 50μM verapamil. The I-V relationship of the C1 neurone was greatly flattened in the presence of 50μM verapamil. B, graph showing the difference between currents in control and 50μM verapamil solutions. Unlike the differences observed in Co and low Ca solutions, the difference in 50μM verapamil was not bell shaped and suggested that other outward currents, in addition to the Ca-dependent K current were blocked.


A
1.6
1.2
0.8
0.4
current (μA)
-40
0
40
80
120
160
200
Vm (mV)


B
0.6
0.4
0.2
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

the effects of 5 and 50μM verapamil on the currents recorded from the C1 neurone is shown in figure 3.30. Whilst 5μM verapamil only produced a very slight reduction of the currents, 50μM verapamil greatly reduced the outward currents, even at very depolarised potentials (+150mV), where the Ca-dependent component of the outward current should be negligible. This suggested that 50μM verapamil had an nonspecific effect, blocking outward currents which did not depend on the influx of Ca^{2+} ions.

The results of the experiments with Co and Ca free solutions show that the shallow N shaped component of the I-V curve of the C1 neurone is most likely to be the Ca-dependent K current described by Meech and Standen (1975). The effect of 5-HT on this component of the I-V curve was therefore tested. In 5 out of 12 experiments 5-HT (50μM) caused a flattening of the shallow N shaped I-V relationship of the C1 neurone. In the remaining 7 experiments, no effect of 5-HT on the I-V curve at very depolarised potentials was observed. In 3 of the cells in which a flattening of the I-V relationship was apparent, the difference between the records in 5-HT and control solutions was a clear bell shaped relationship as seen with Ca free solution. Figure 3.31 shows one such experiment. The similarity between the effect of the 5-HT solution and Ca free solution suggested that, in this cell, 5-HT reduced the Ca-dependent K current. In 2 other experiments where 5-HT flattened the I-V curve of the C1 neurone a plot of the difference between 5-HT and control records was only slightly bell shaped and resembled the effect of 5μM verapamil (see figure 3.28). These results suggested that 5-HT may have effected another current in addition to the Ca-dependent K current in these cells.

Figure 3.30

Comparison of the effects of 5 and 50μM verapamil on currents elicited from the C1 neurone by 100ms depolarising steps from a holding potential of -50mV. A, currents recorded in control and 5μM verapamil solutions. No pronounced N shape was observed under control conditions in this C1 neurone. In the presence of 5μM verapamil the outward currents elicited were only slightly reduced. The currents shown correspond to command potentials to, 30, 50, 70, 90, 110, 130, 150 and 170mV in ascending order. B, currents elicited from a different C1 neurone by the same depolarising steps. Again no pronounced N shape was observed under control conditions. In the presence of 50μM verapamil the outward currents were markedly reduced when measured at the end of the step. The shape of the currents was also changed. They reached a peak in the first 5ms then decayed throughout the following 50ms to a lower steady state level. The final current corresponding to the step to 170mV is missing in the 50μM verapamil record because the cell died on this step.

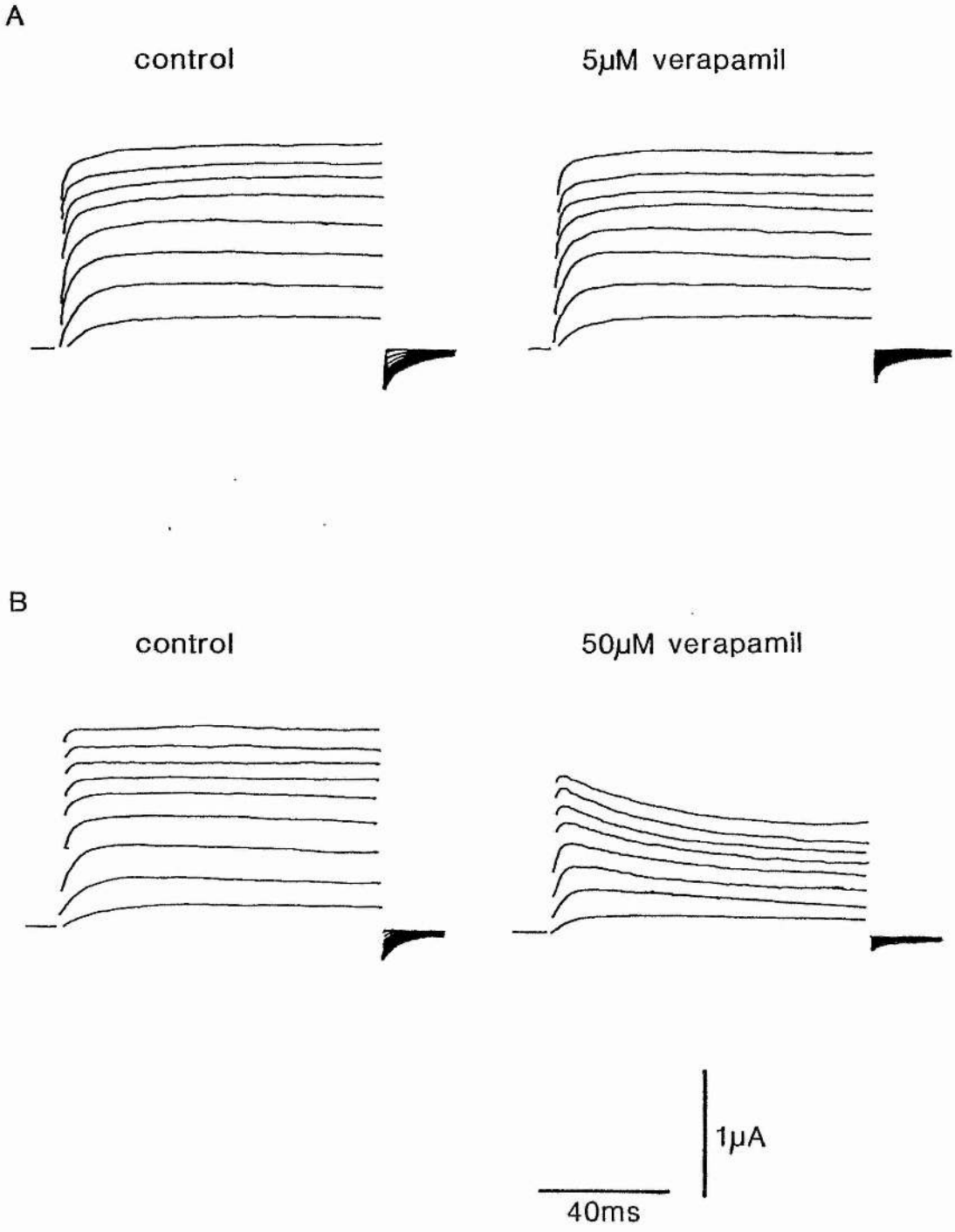
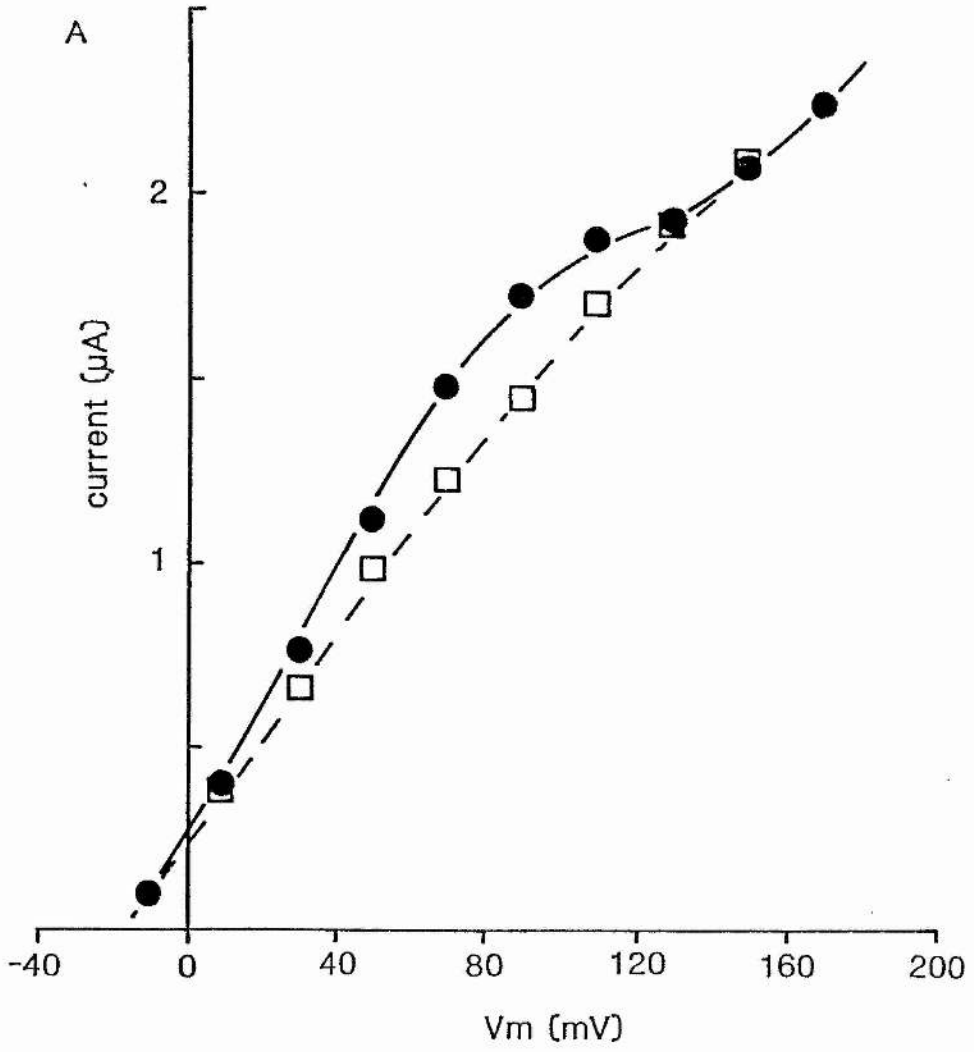
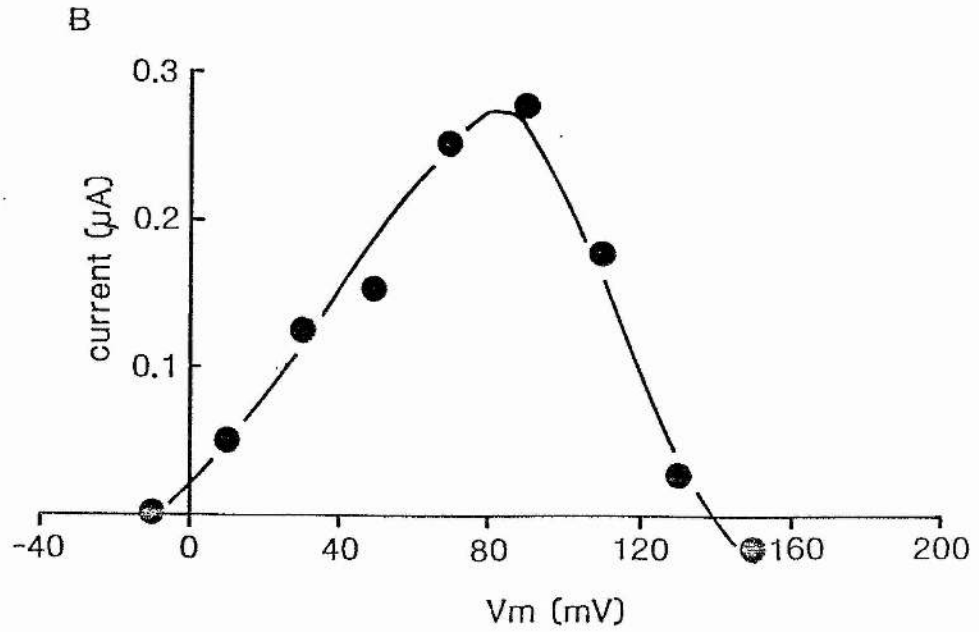

A
control
5μM verapamil
B
control
50μM verapamil
1μA
40ms

Figure 3.31

Effect of 50μM 5-HT on the I-V relationship of the C1 neurone. A, I-V relationships obtained by stepping Vm from a holding potential of -50mV to depolarised potentials for a duration of 100ms as before. ●- Control. □- 50μM 5-HT. The N shape of the I-V relationship is slightly flattened in the presence of 50μM 5-HT. B, graph showing the difference between currents in control and 50μM 5-HT solutions on an expanded scale. The difference is roughly bell shaped, with a peak at about +80mV, suggesting that 5-HT reduces a Ca-dependent K current.


A
2
1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)


B
0.3
0.2
0.1
current (μA)
-40
0
40
80
120
160
200
Vm (mV)

The lack of effect of 5-HT on the I-V curves of some C1 neurones was difficult to explain, since 5-HT always induced an inward current when the C1 neurone was held at steady state depolarised potentials, between -40 and -10mV. Tests using methylene blue dye suggested that the perfusion system caused an adequate exchange of solution. In experiments where the concentration of 5-HT was increased to 500μM, some cells still showed no flattening of the I-V relationship.

3.10.2 EFFECT OF VERAPAMIL ON THE RESPONSE TO IONOPHORESED 5-HT

The Ca-dependency of the 5-HT response was further investigated using a single electrode voltage clamp technique to study the response to ionophoresed 5-HT in C1 neurones held at a steady state depolarised level. The following sections describe some experiments performed using this technique. The Dagan single electrode voltage clamp system was suitable for these experiments since the current required to be passed was less than 25nA and the responses studied were relatively slow.

Previous experiments had shown that the Ca current blocking agent Co (1mM) markedly reduced the size of the inward current response to 5-HT in a voltage clamped C1 neurone (Barnes, Cottrell and Dunbar, in preparation). The effect of the organic Ca current blocking agent, verapamil on the 5-HT response of the C1 neurone was investigated. The experiments above suggested that 50μM verapamil blocked other outward currents in addition to the K current activated by Ca influx during depolarisation. At this concentration verapamil would be expected to

significantly reduce the Ca current of Helix neurones (Akaike, Brown, Nishi and Tsuda, 1981). However, nonspecific actions of verapamil have also been reported (Gola and Ducreux, 1985). Earlier experiments had shown that 10μM verapamil had no effect on the response to 5-HT (Barnes, Cottrell and Dunbar, in preparation). In the experiments performed in this study, perfusing the preparation with a solution containing 50μM verapamil had no effect on the response to ionophoresed 5-HT (see figure 3.32). The lack of effect of this concentration of verapamil (which would be expected to significantly reduce the Ca current) suggested that the response to 5-HT in the C1 neurone may not depend on the influx of Ca^{2+} ions during depolarisation.

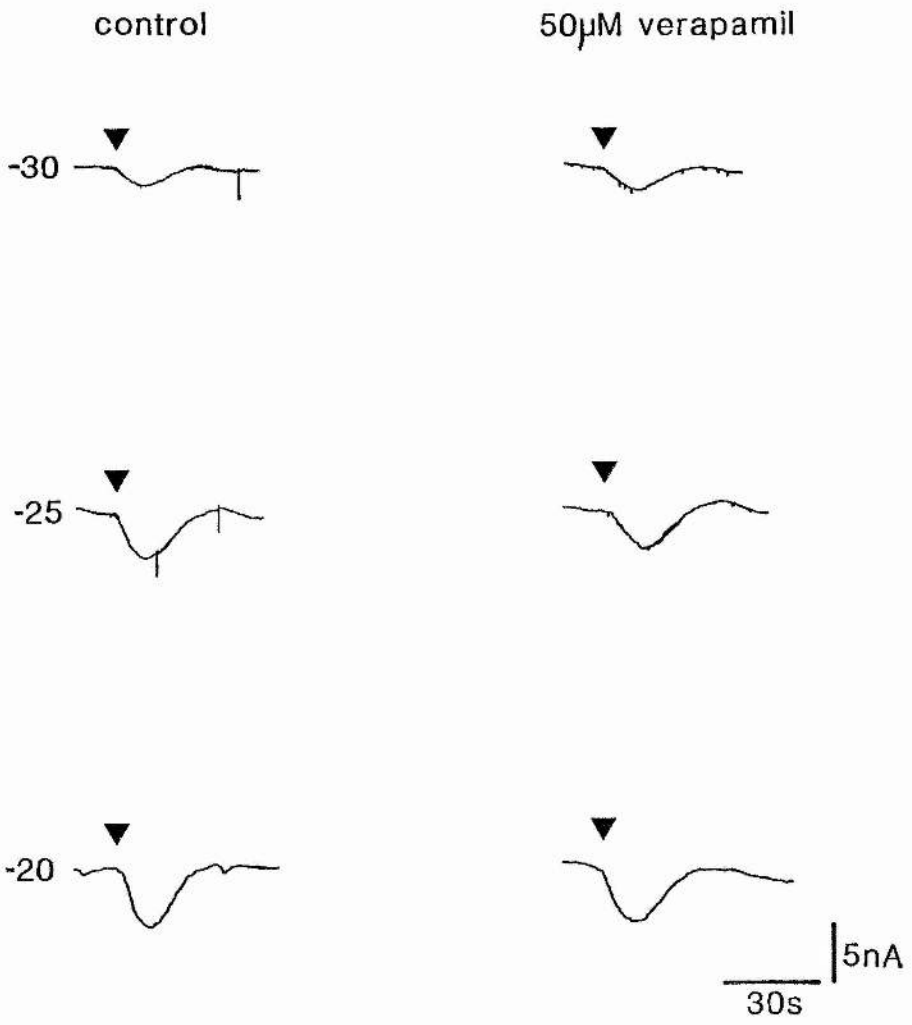
3.10.3 EFFECT OF PROLONGED EGTA INJECTION

Intracellular injection of EGTA was made by ionophoresis from double barrelled θ electrodes. Both barrels of the electrode contained 0.25M EGTA dissolved in 0.5M KOH to a pH of 7.0. The use of double barrelled electrodes prevented artefacts due to the injection of OH^- ions. EGTA was injected into the cell by passing a current of 100nA between the two barrels for about 15min.

Prolonged intracellular injection of the Ca chelating agent, EGTA, would be expected to reduce the Ca-dependent K current by chelating Ca^{2+} ions which entered the cell during depolarisation. Injection of EGTA has been used to block the Ca-dependent K current in molluscan neurones (Deterre, et al, 1982; Kehoe, 1985b). A response, which was due to a decrease of this current, should therefore, be reduced after injection of EGTA. A flattening of the I-V relationship of the cell

Figure 3.32

The lack of effect of 50μM verapamil on the response to 5-HT in a voltage clamped C1 neurone. Responses to ionophoretically applied 5-HT (▼) are shown from a C1 neurone at holding potentials of -30, -25 and -20mV, in control conditions and in the presence of 50μM verapamil. The response to 5-HT was unaffected in saline containing 50μM verapamil. The occasional downward spikes seen in these traces are probably unclamped axonal action potentials.


control
50μM verapamil
-30
-25
-20
5nA
30s

would also be expected under these conditions. No consistent effect of EGTA injection on the I-V relationship of the C1 neurone between -80 and -10mV was observed. This may be because at these potentials the Ca-dependent component of the outward current was not substantial. In the example shown in figure 3.33,A, no effect of EGTA injection on the I-V curve was apparent.

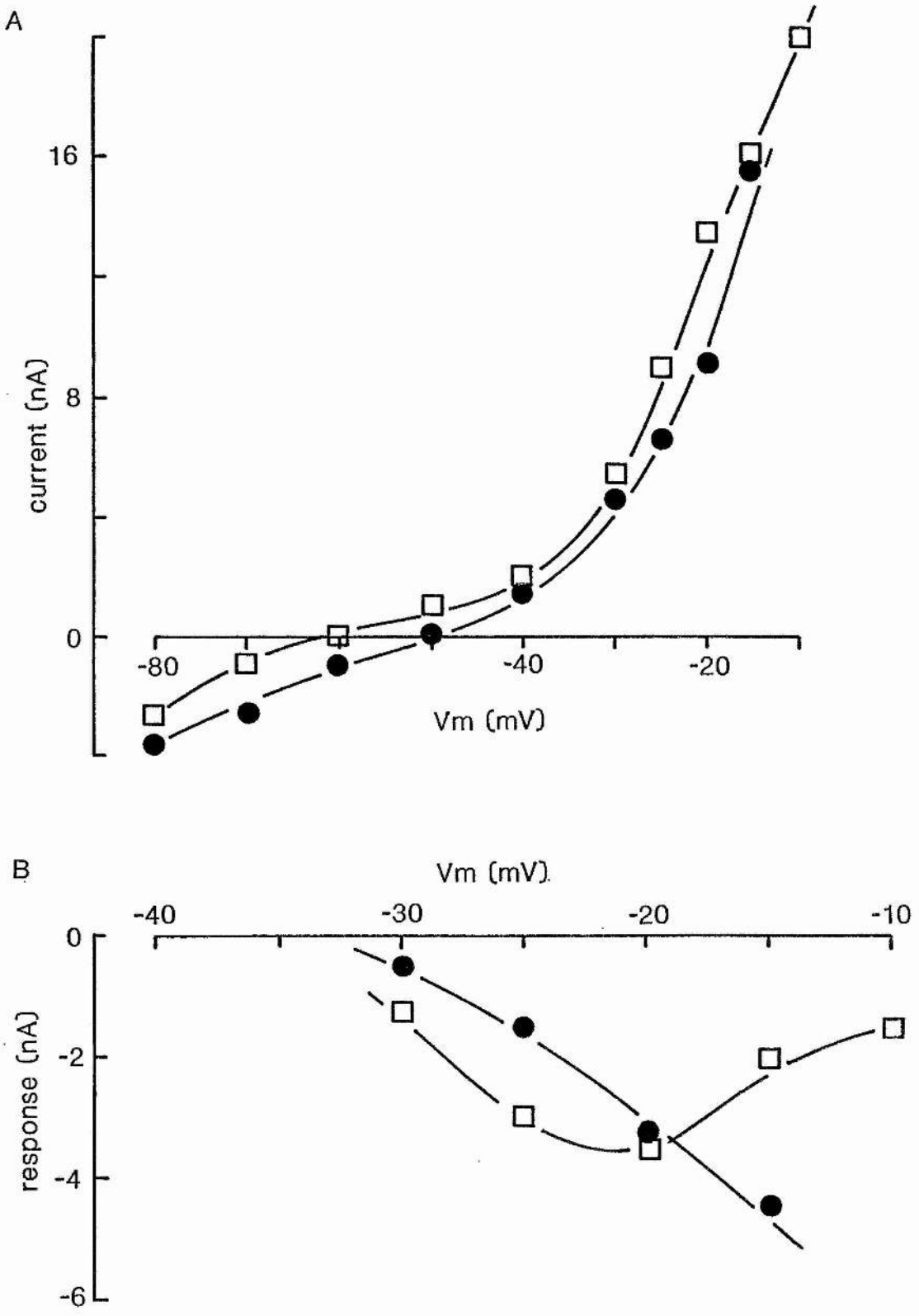
Despite the lack of effect of EGTA injection on the I-V relationship of the C1 neurone, an effect on the response to ionophoresed 5-HT was observed. In 7 out of 10 experiments the response to 5-HT at -10mV was reduced. However, often, as in the example shown in figure 3.33, the response to 5-HT at less depolarised potentials was either increased or unaffected.

3.10.4 EFFECT OF INTRACELLULAR Ca INJECTION

If the response to 5-HT was due to a decrease in a Ca-dependent K current, activating this current by increasing the intracellular level of Ca would be expected to enhance the response. Ca^{2+} ions were injected into the C1 neurone by ionophoresis from a single barrelled electrode whilst the cell was held under voltage clamp at -40 or -30mV. This evoked an outward current. The injection of Ca^{2+} ions was sometimes repeated in an attempt to obtain a stable Ca-dependent outward current. However, in most cases the outward current rapidly decayed after an initial rise. This was probably due to intracellular Ca sequestration (Rose and Lowenstein, 1975). The outward current evoked by Ca injection in the C1 neurone has been shown to be a Ca-dependent K current (Cottrell, 1982c; Cottrell, Davies and Green,

Figure 3.33

The effect of prolonged intracellular EGTA injection on the I-V relationship of the C1 neurone and the response to ionophoresed 5-HT. A, I-V relationships recorded in control conditions (●) and after 15min injection of EGTA (□). EGTA was injected by ionophoresis, from a double barrelled electrode containing 0.25M EGTA, using a current of 100nA. In this example very little change in the I-V curve after the injection of EGTA was observed. B, a plot of the 5-HT response amplitude against holding potential for the same C1 neurone before (●), and after (□) EGTA injection.


A
16
8
0
current (nA)
-80
-40
-20
Vm (mV)
B
Vm (mV)
-40
-30
-20
-10
0
-2
-4
-6
response (nA)

1984).

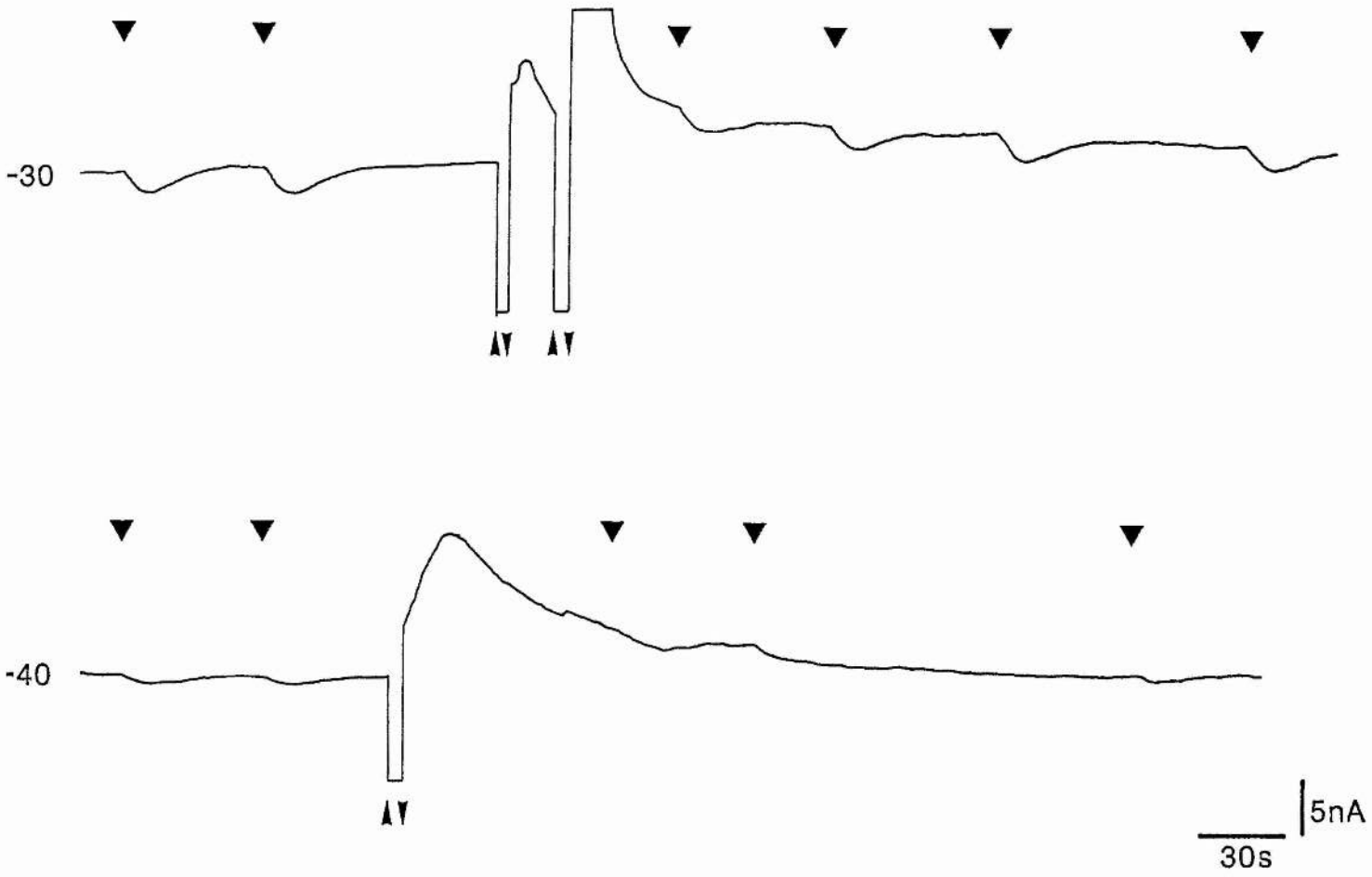
The response to ionophoresed 5-HT was examined before and after the injection of Ca^{2+} ions. In the 8 experiments performed, no enhancement of the 5-HT response after the injection Ca^{2+} ions was observed. Examples of two experiments, in which no clear increase in the amplitude of the 5-HT response was observed after Ca injection, are shown in figure 3.34. In both of these examples larger responses to 5-HT could be evoked by a further depolarisation of the cell.

3.10.5 EFFECT OF A LOW Ca SOLUTION

In a further attempt to determine the Ca-dependency of the 5-HT response, the effect of perfusing the preparation in a nominally zero Ca solution was examined. Nominally zero Ca solutions contained no added $CaCl_2$ and an extra 7mM $MgCl_2$ or 14mM NaCl to maintain osmolarity. Results from these experiments were confusing since the response was increased if the cell was held under voltage clamp, but was irreversibly diminished if the low Ca solution was perfused through while the cell was under current clamp conditions. These conflicting results may have been due to problems with a change in the surface charge of the membrane when the cell was perfused in a low Ca solution. If the K current reduced by 5-HT was dependent on the influx of Ca^{2+} ions for activation, one would expect the 5-HT response to be reduced when the extracellular Ca concentration was lowered. Experiments described above on the Ca-dependent N shape of the I-V curve suggested that perfusing the preparation with a nominally zero Ca solution significantly reduced the Ca-dependent K current.

Figure 3.34

Intracellular Ca injection experiments. Responses to ionophoresed 5-HT (▼) were obtained before and after Ca injection. Ca^{2+} ions were injected from a single-barrelled, $CaCl_2$ electrode by ionophoreses, using a 60nA current. The duration of Ca injections are shown by the arrows. The upper trace shows a recording from a C1 neurone voltage-clamped to -30mV. No significant change in the amplitude of the 5-HT response was observed after Ca injection. The lower trace shows a recording from a different C1 neurone, voltage-clamped to -40mV. Ca injection caused a small increase of about 3nA in the outward current in this cell, but did not increase the amplitude of the very small 5-HT response.


-30
-40
5nA
30s

3.11 INVOLVEMENT OF AN INTRACELLULAR MESSENGER IN THE 5-HT RESPONSE

The ability of 5-HT to reduce the activity of K channels when applied to the C1 neurone, from outwith the patch pipette, suggested the involvement of an intracellular messenger in the response. The possible role of a cyclic nucleotide in mediating the response was investigated using phosphodiesterase inhibitors. These substances inhibit the breakdown of cyclic nucleotides by interfering with the phosphodiesterase enzyme.

Perfusing the preparation with 10μM theophylline induced an inward current in a voltage clamped C1 neurone, held at a depolarised potential. During the theophylline induced inward current the response to ionophoretically applied 5-HT was attenuated (see figure 3.35). Exposure of the preparation to 0.1mM IBMX also produced an inward current in a C1 neurone held at a depolarised potential. This effect was seen as a flattening of the I-V curve and was accompanied by a reduction in the size of the 5-HT response (figure 3.36). These results may be explained in terms of a basal level of cyclic nucleotide production in the C1 neurone. The action of theophylline and IBMX may be to increase the basal level of cyclic nucleotides. The cyclic nucleotides may then reduce the K current of the C1 neurone.

In one C1 neurone, in which no flattening of the I-V curve was apparent, the response to 5-HT was prolonged during perfusion with 0.1mM IBMX (figure 3.37). The increase in the duration of the response could be due to the prevention of the breakdown of a, 5-HT-induced,

Figure 3.35

Effect of 10μM theophylline on the inward current response to 5-HT. The upper trace shows control responses to ionophoretically applied 5-HT (▼) in a C1 neurone, voltage-clamped to -30mV. The middle trace shows two responses in the presence of theophylline. The first response was obtained 3.5min after changing the perfusing solution to theophylline and the second response was obtained 5min after changing solutions. Perfusion with theophylline induced an inward current of -2.5nA. Recovery of the 5-HT response after around 10min wash in control solution is shown in the lower trace.

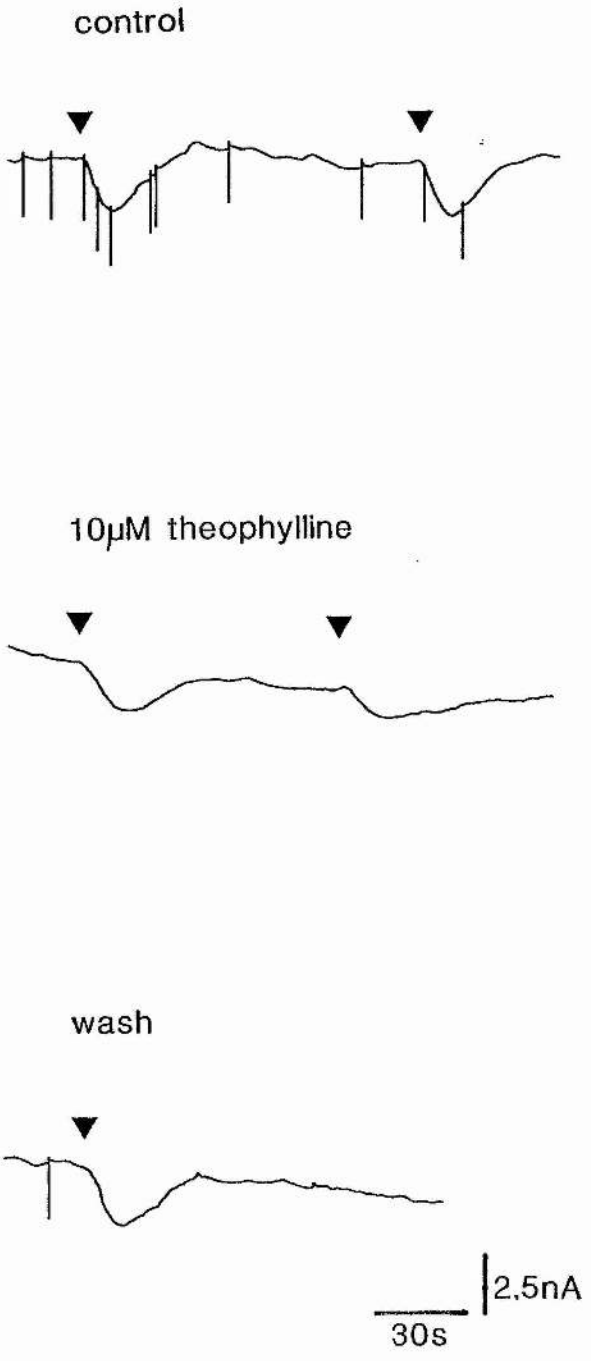

control
10μM theophylline
wash
2.5nA
30s

Figure 3.36

Effect of 0.1mM IBMX on the I-V relationship of the C1 neurone and the inward current response to 5-HT. A, I-V relationships of the C1 neurone in control (●) and IBMX (□) solutions. A slight flattening of the I-V curve at Vh= -25mV in the presence of IBMX is apparent in this example. B, reduction in the amplitude of the response to ionophoresed 5-HT (▼) on perfusion with 0.1mM IBMX for 5min. Upper record, Vh= -30mV. Lower record, Vh= -25mV. A recovery of the 5-HT response was seen on washing through control solution for 10min. These records were obtained from the same cell as in part A.

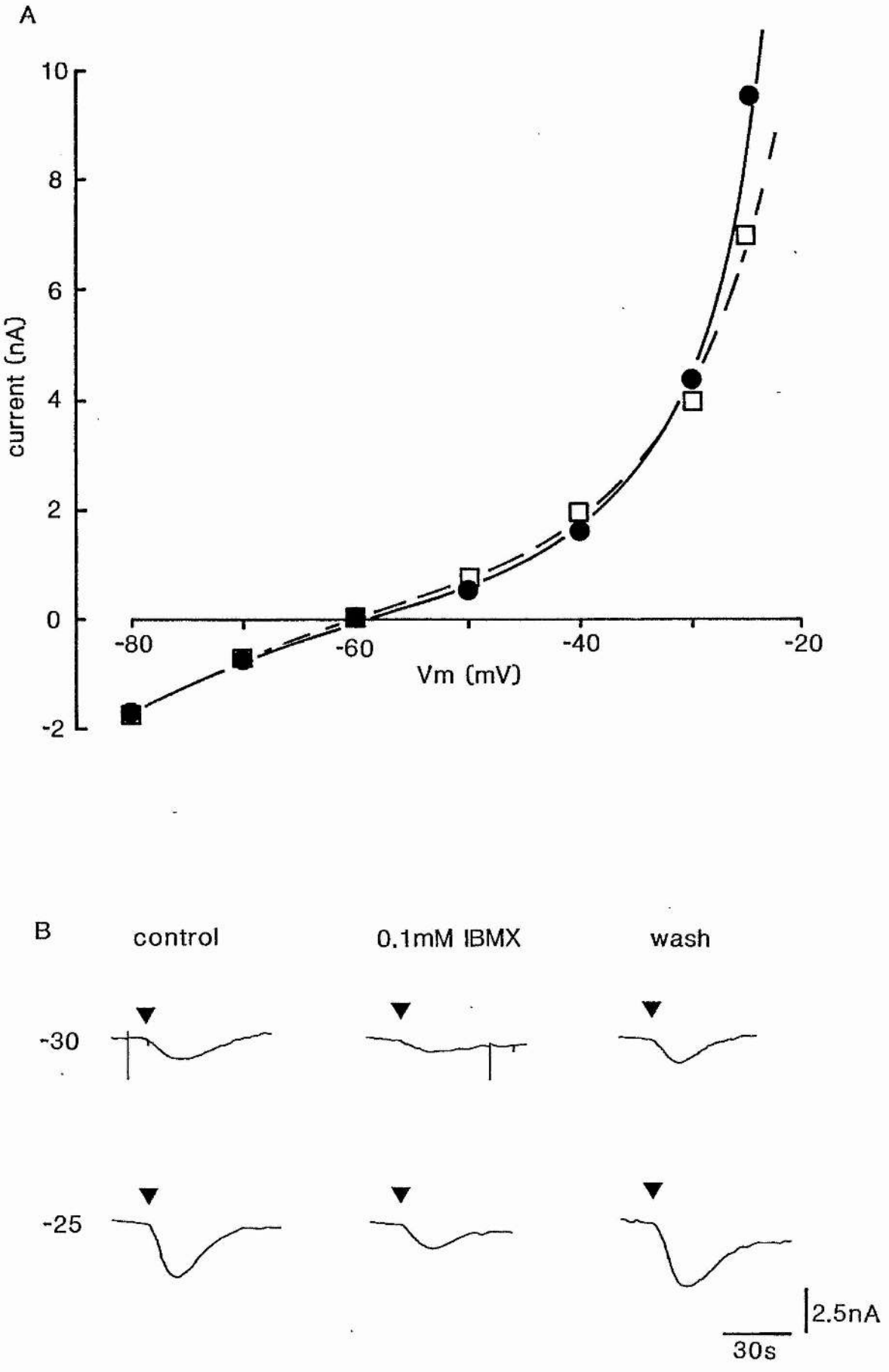
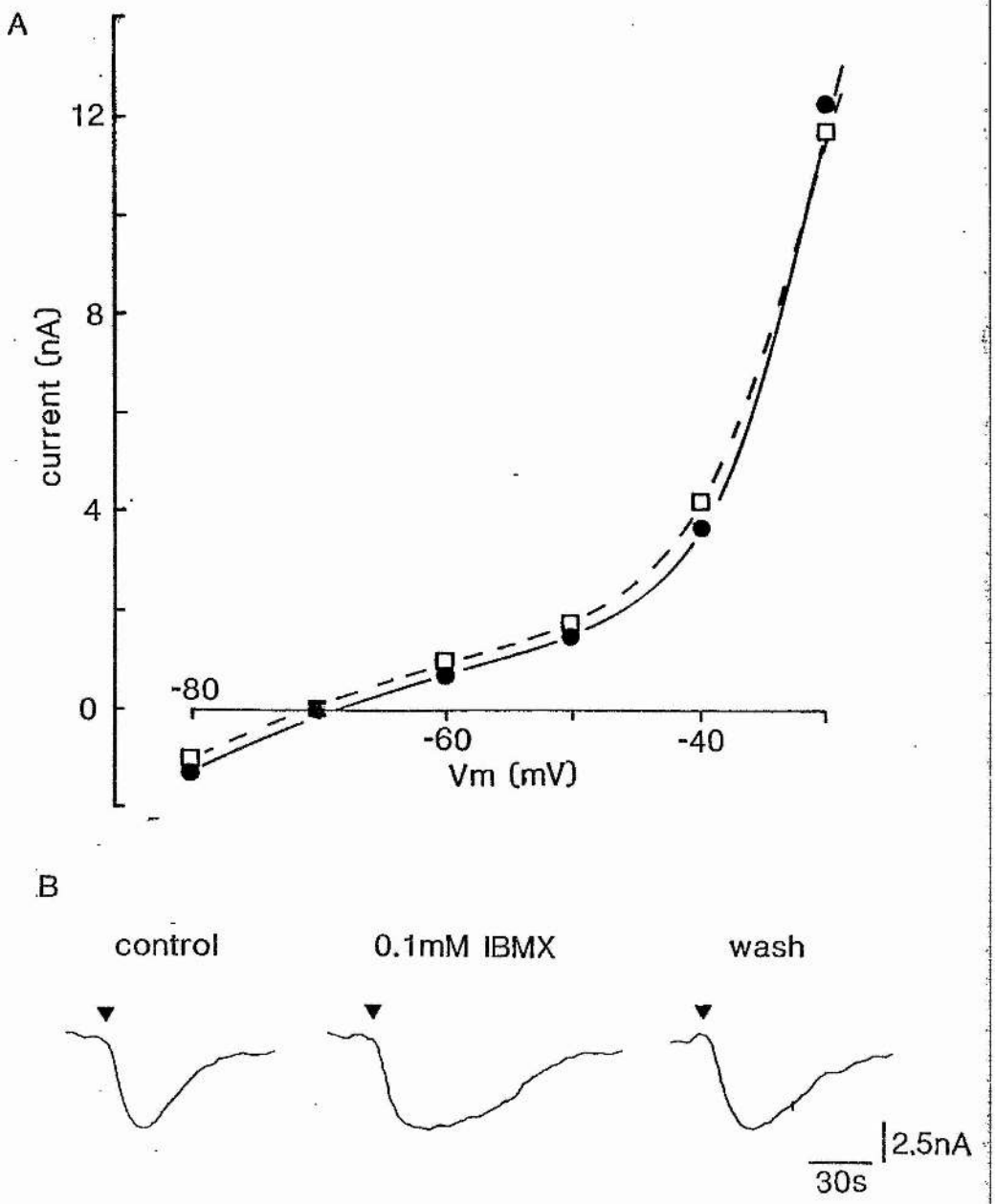

A
10
8
6
4
2
0
-2
current (nA)
-80
-60
-40
-20
Vm (mV)
B
control
0.1mM IBMX
wash
-30
-25
2.5nA
30s

Figure 3.37

An example were 0.1mM IBMX had no effect on the I-V relationship of the C1 neurone, but caused an increase in the duration of the response to ionophoresed 5-HT . A, I-V relationships of the C1 neurone in control (●) and IBMX (□) solutions. B, responses to ionophoresed 5-HT (▼) in the same C1 neurone as in part A above. Vh=-30mV. The response to 5-HT was prolonged after 5min perfusion with 0.1mM IBMX. This effect was partially reversible on washing through with control solution.

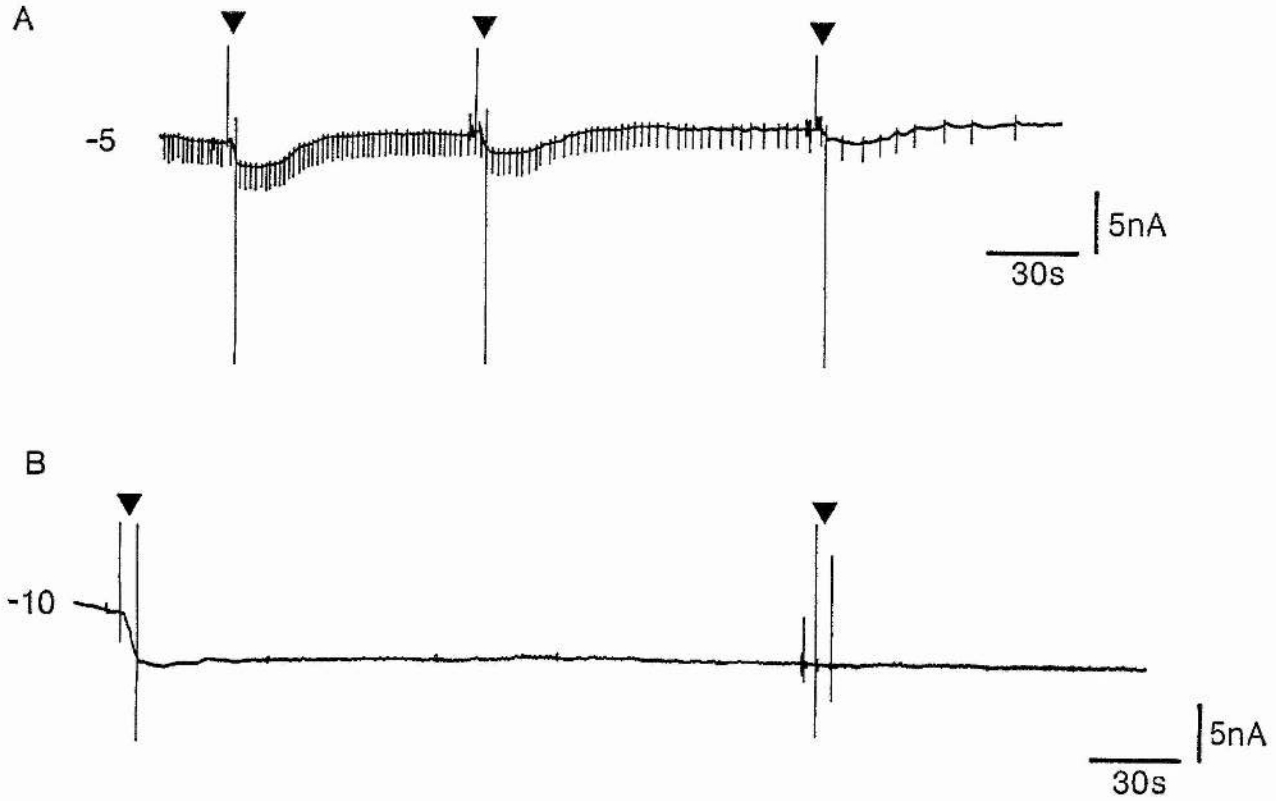

A
12
8
4
0
current (nA)
-80
-60
-40
Vm (mV)
B
control
0.1mM IBMX
wash
2.5nA
30s

increase in cyclic nucleotides. This effect was observed on only one occasion.

Intracellular injection of cAMP has been shown to decrease the number of K channels open in cell-attached patches of Aplysia sensory neurones (Siegelbaum, et al, 1982). cAMP may mediate the 5-HT response of these cells (Brunelli, et al, 1976). The 5-HT response in some Helix neurones has also been reported to be mediated by cAMP (Deterre, et al, 1981;1982). The possibility that cAMP may be capable of mediating the 5-HT response in the C1 neurone was investigated by injecting cAMP into voltage clamped C1 neurones. Cyclic AMP was injected by ionophoresis from double barrelled θ glass electrodes. Both barrels were filled with 0.1M cAMP dissolved in 0.1M KOH to pH 7. A current of 80nA was passed between the barrels to inject cAMP into the cell. Figure 3.38 shows the inward currents induced by 3 or 5s injections of cAMP. This inward current was not apparent at hyperpolarised potentials, but was only observed in cells held at depolarised potentials. However, it was not possible to obtain a relationship for the response amplitude against holding potential because the response to cAMP declined with repetitive injections (see figure 3.38,A). Moreover, if the duration of the injection current was longer than 4 or 5s, a prolonged inward current, as seen in figure 3.38,B, was produced. Further injection of cAMP during this prolonged inward current did not elicit a response. The maintained inward current, and decline of the response with repeated injection, suggested that the actions of cAMP and 5-HT may be different.

Figure 3.38

Response to intracellular cAMP injection. Upper trace - recording from a C1 neurone at a holding potential of -5mV. cAMP was injected (▼) by passing an 80nA current for 3s. Injection of cAMP elicited an inward current, which declined in amplitude with repeated injections. Lower trace - recording from a different C1 neurone at a holding potential of -10mV. In this record cAMP was injected (▼) using a 80nA current for a duration of 5s. The first injection produced a prolonged inward current response, which did not recover fully over a duration of 6min. A further injection of cAMP, during this period failed to elicit an inward current response.


A
-5
5nA
30s
B
-10
5nA
30s

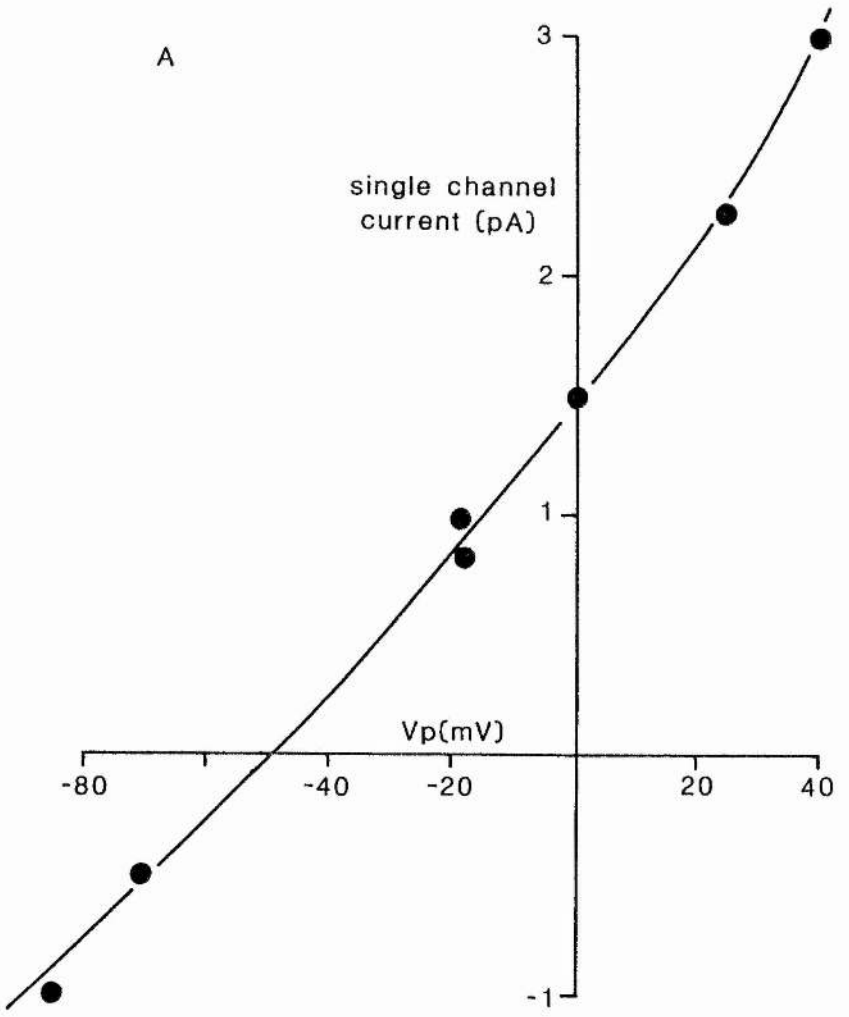
3.12 SINGLE CHANNEL CURRENTS OF THE A AND M NEURONES AND THE EFFECT OF 5-HT

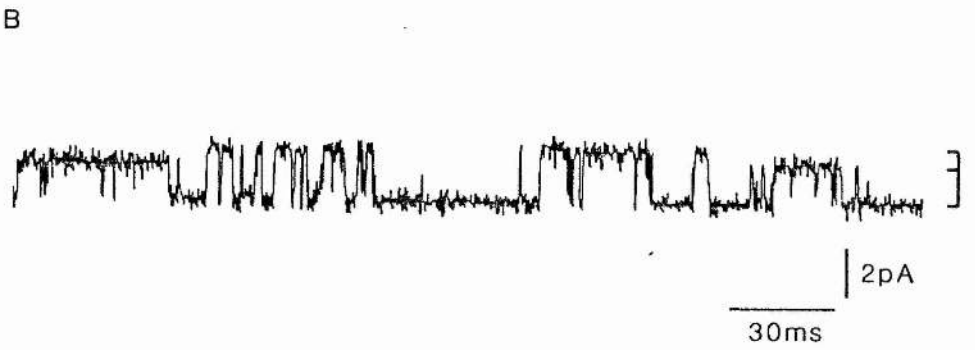
A voltage-dependent response to 5-HT, similar to that seen in the C1 neurone, was earlier shown to be evoked in the A neurone of the buccal ganglion by, both synaptically released, and exogenously applied, 5-HT (Cottrell, 1971;1981;1982b). A similar response was also found in the M neurone of the buccal ganglion, in conjunction with a fast Na-dependent depolarisation (Cottrell, 1982b). It was therefore of interest to determine if the K channels present in the C1 neurone were present in the A and M neurones, and if their activity was affected by the application of 5-HT.

Outward channel currents were recorded from cell-attached patches of the A neurone. At least two sizes of outward channel currents were observed in patches from the A neurone. An example of these is shown in figure 3.39,B. The smaller of these unitary outward currents were more frequently observed and this enabled an I-V relationship of them to be constructed. Figure 3.39,A shows an I-V relationship of these channels. Unlike the unitary outward currents recorded on the C1 neurone, these single channel outward currents did reverse at hyperpolarised patch potentials. A line draw by eye through the data points suggested a reversal potential of about -50mV. This is more positive than the calculated E_K value, of -74.4mV, assuming an internal K concentration of 98mM. This I-V relationship could therefore not be fitted by the theoretical relationship calculated from the GHK equation for a K current and it was concluded that these channels were different

Figure 3.39

Unitary outward currents recorded from a cell-attached patch of an A neurone. A, I-V relationship of the commonly observed single channel currents. The line was fitted to the points by eye and shows a reversal of the unitary outward currents at a patch potential of about -50mV. Vm, measured at the end of this experiment, using the patch pipette, was -49mV. B, record from a different A neurone, showing the existence of two sizes of unitary outward currents on a cell-attached patch. Vp=0mV in this record. The smaller outward currents of about 1.5pA at this potential are equivalent to those represented in the I-V relation of part A. Vm in this cell was recorded as -45mV at the end of the experiment.


A
single channel
current (pA)
3
2
1
-1
Vp(mV)
-80
-40
-20
20
40


B
2pA
30ms

from the 14pS and 54pS K channels recorded in cell attached patches of the C1 neurone. The I-V relationship of these channels was more linear and the slope conductance at +10mV was around 37pS. The I-V relationship of these channels suggested that the current flow was not carried solely by K^+ ions. Since their reversal potential was nearer to E_{Cl} than E_K, the single channel currents recorded in the A neurone may have been Cl channels. Alternatively, they may be a type of channel which is largely selective to K^+ ions, but also allows the flow of an appreciable amount of Na^+ ions.

Application of 10^{-4}M 5-HT onto the A neurone, whilst recording from a cell-attached patch, produced an increase in the activity of these channels in 4 out of 6 experiments (see figure 3.40). An increase in the activity of outward channels upon application of 5-HT was not expected from the results of intracellular recording, in which a depolarising response to 5-HT is seen. The increased activity of these channels should be manifest as a hyperpolarisation at depolarised potentials. The possible explanation for these results shall be reviewed in the discussion.

Single channel currents were recorded from cell-attached patches of the M neurone. An I-V relationship for these unitary currents is shown in figure 3.41. This relationship could be fitted by the theoretical curve calculated from the GHK equation for a K current. The value of the permeability calculated from the data points was $1.2 \times 10^{-13} cm.^3 s^{-1}$. This value lies between the pK values calculated for the 14pS and 54pS unitary K currents recorded from cell-attached patches of the C1 neurone. However, this was the only I-V relationship

Figure 3.40

Increased activity of unitary outward currents of the A neurone in the presence of 5-HT. Records are from a cell-attached patch, at a patch potential of 0mV. The upper trace shows the control record before the application of 5-HT. The middle trace shows the recording seconds after application of 10^{-4}M 5-HT by microperfusion. These recordings are from the same patch as in part B of figure 3.39. The smaller (1.5pA) channels were activated by application of 5-HT. The lower trace is a section of recording 1min after removal of the 5-HT microperfusion pipette, showing a decrease in channel activity back to control levels.

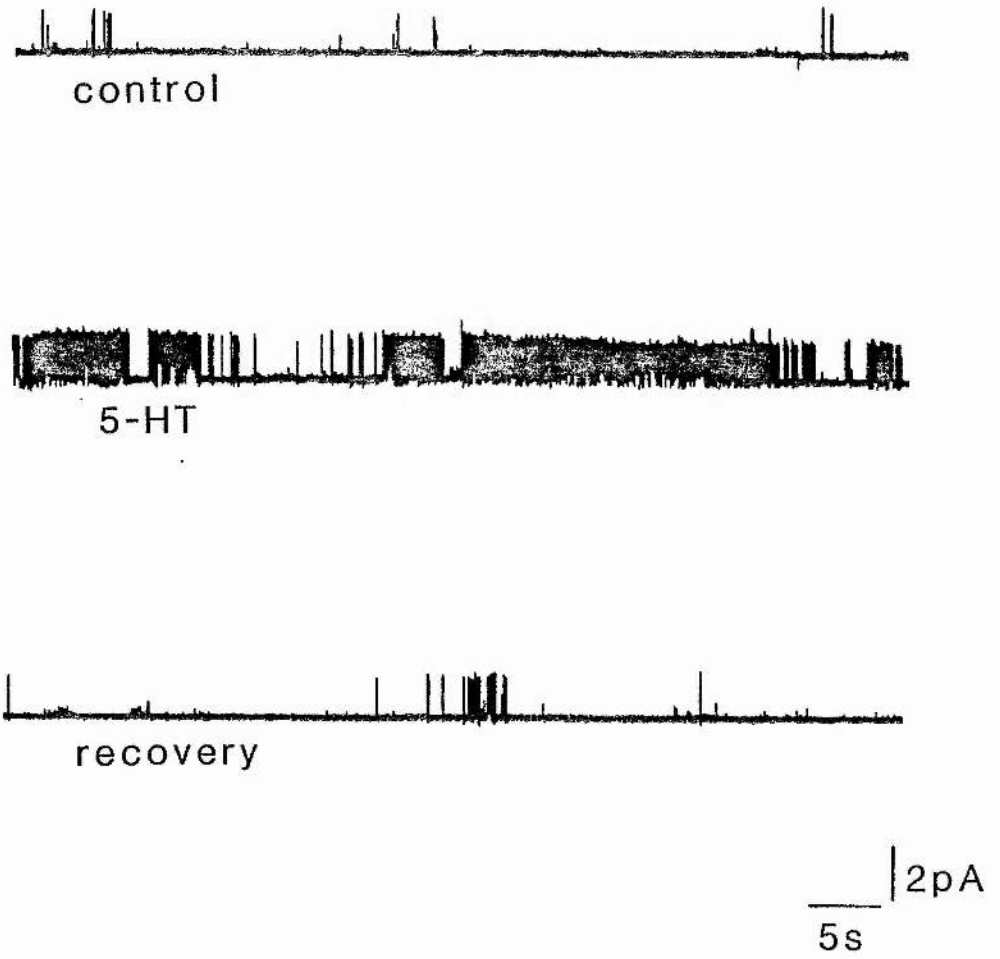

control
5-HT
recovery
2pA
5s

Figure 3.41

I-V relationship of unitary outward currents recorded from a cell-attached patch of the M neurone. The data have been fitted by the theoretical relationship, calculated from the GHK equation for a K current, using a permeability value of $1.24 \times 10^{-13} cm^3.s^{-1}$. Vm measured at the end of the experiment with the patch pipette was -50mV. The theoretical fit was made assuming an intracellular K concentration of 98mM.

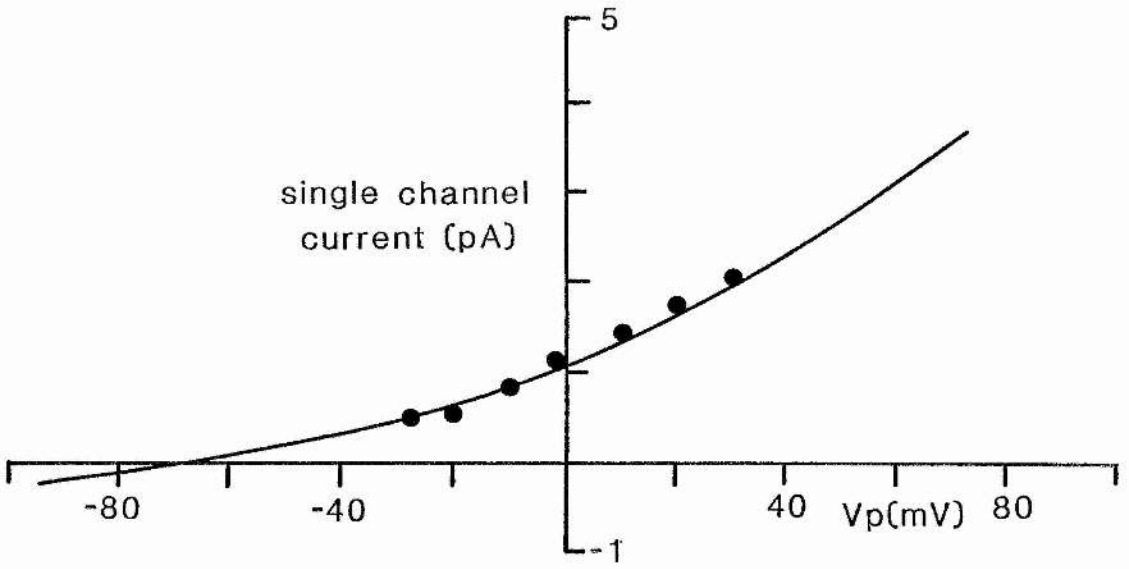
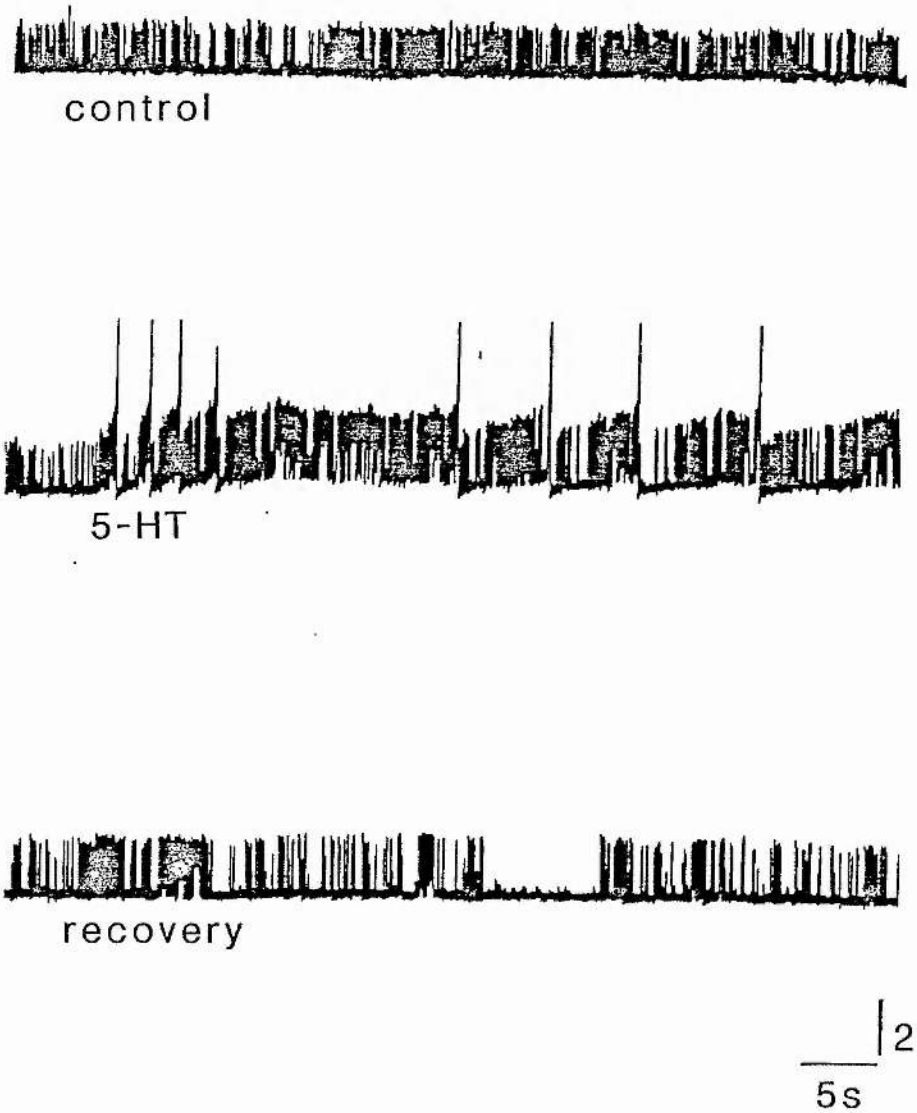

5
single channel
current (pA)
-80
-40
40
Vp(mV)
80
-1

Figure 3.42

The effect of 5-HT on unitary outward currents recorded from a cell-attached patch of the M neurone. The upper trace is the recording under control conditions at a patch potential of +15mV. The middle trace is a section of recording 30s after microperfusion with 5-HT. An increase in the channel amplitude and the appearance of extracellularly recorded action potentials were observed. The lower trace shows a section of recording 4min after removing the 5-HT pipette. The channel amplitude is still increased above control levels in this record.


control
5-HT
recovery
2pA
5s

obtained for unitary currents from a cell which was identified with reasonable certainty as the M neurone.

Application of 5-HT onto the M neurone, whilst recording single channel currents from a cell-attached patch, caused a depolarisation of the M neurone. This was evident due to the appearance of action potentials, which could be detected extracellularly by the patch pipette. The accompanying depolarisation also caused an increase in the amplitude of the unitary currents (figure 3.42). The membrane potential was not monitored during this recording. The effect of 5-HT in this cell demonstrates the unsuitability of the cell-attached mode of the patch clamp technique for studying transmitter effects where a change in the resting membrane potential occurs.

3.13 SUMMARY OF THE RESULTS

A patch clamp study of unitary outward currents in the C1 neurone was made. Single channel K currents of 14pS and 54pS were recorded from cell-attached patches of the C1 neurone. 10^{-4}M 5-HT caused a reduction in the total open time of the larger channels when applied to the cell from outwith the patch pipette. This indicated the involvement of an intracellular messenger.

Both Ca-dependent and Ca-independent K channels were recorded in isolated inside-out patches from the C1 neurone. These had mean slope conductances of 50pS and 79pS, respectively, in physiological K gradients. It was uncertain which of these types of channel corresponded to the 54pS K channel, recorded from cell-attached

patches, and whose activity was decreased by the application of 5-HT.

Voltage clamp studies were undertaken to investigate if the inward current response to 5-HT was due to a decrease in a Ca-dependent K current or a voltage-dependent K current. The results of voltage clamp experiments were complicated, suggesting in some cases a Ca-dependency and in other cases no apparent Ca-dependency. The Ca-dependent component of the I-V curve was reduced by 5-HT in some experiments. Injection of EGTA reduced the response to 5-HT at potentials more depolarised than -15mV. However a concentration of verapamil which should significantly reduce the Ca current had no effect on the 5-HT response. Injection of Ca^{2+} ions into the cell (to increase the Ca-dependent K current) also had no effect on the inward current response to 5-HT.

CHAPTER 4

DISCUSSION

DISCUSSION

4.1 GENERAL PROPERTIES OF SINGLE CHANNEL OUTWARD CURRENTS OF THE C1 NEURONE

Unitary outward currents normally observed in cell-attached patches of the C1 neurone could be classified into two types on the basis of their I-V relationships. For both types of channel these relationships were non-linear and could, in general, be fitted with the theoretical relationship calculated from the GHK equation for a K current. The channels were identified on the basis of permeability values or conductance. Since the I-V relationships were non-linear, the slope conductance at +10mV was calculated by fitting a tangent (by eye) to the curve at this point. The values of slope conductance (g), under normal physiological conditions, and permeability (P_K) are summarised below.

	g(pS)	$P_K(cm.^3s^{-1})$
small channels	14.2 ± 2.1	$6.7\pm1.4\times10^{-14}$
large channels	53.8 ± 3.3	$2.3\pm0.4\times10^{-13}$

The channels in the cell-attached patch were then referred to as 14pS and 54pS channels.

The GHK equation, used to fit the data, assumes a constant field model for membrane rectification (Goldman, 1943; Hodgkin and Katz, 1949). This theory assumes a linear potential drop across the membrane and an independent movement of ions. The penetration of the ions into the membrane is independent of the electric field, whilst the movement through the membrane depends on the linear

potential drop across the membrane. In the experiments reported here certain assumptions were made in order to calculate the theoretical relationship of the GHK equation. A value of 98mM was assumed for the intracellular K concentration (cf. Alvarez-Leefmans and Gamino 1982; Cottrell, Davies and Green, 1984). In experiments in which it was not possible to measure Vm directly, a value of -60mV was assumed for the resting potential of the C1 neurone. This was the mean value from 38 experiments, with a standard deviation of 8mV. Errors in both of the above assumptions may have lead to theoretical relationships which were not true for the conditions of the cell.

The slope conductance of the channels has been quoted throughout. However, it was difficult to accurately fit a tangent to the curve. An alternative to quoting the slope conductance of the channels, at a given potential, would have been to quote the chord conductance from a zero current point (in this case assumed to be E_K) to a given potential. The chord conductances from -75mV (value of E_K assuming an intracellular K concentration of 98mM) to +10mV were in the range of 27 to 34 pS for the large channels and 8 to 9 pS for the small channels. However, it was decided to use the slope conductance at +10mV to compare with previously reported K channels of the C1 neurone (Cottrell, Davies and Green, 1984).

The large conductance channels recorded in this study had a similar conductance and permeability to the channels whose activity was reduced by analogues of the neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2). These channels had a slope conductance at

+10mV of around 52pS and a P_K value of 2.5×10^{-13} cm.^{3}s^{-1} and were probably the same channels as recorded here (Cottrell, Davies and Green, 1984). The small conductance channels recorded by Cottrell, Davies and Green (1984) had a slightly higher conductance than the mean conductance of the small channels studied here (19pS compared to 14pS). This may be due to the difficulties in measuring these low conductance channels accurately.

4.2 IONIC NATURE OF THE UNITARY OUTWARD CURRENTS

The unitary currents recorded from cell-attached patches of the C1 neurone were outwards at all potentials at which they were observed (i.e., Vp of -30 to +60mV). Since both Na^+ and Ca^{2+} ions have their driving forces directed inwards at these potentials, these outward current events could not have been due to the flow of Na^+ or Ca^{2+} ions. Assuming a value of 4.9mM for the internal Na concentration and 10^{-7}M for the internal free Ca concentration (cf Meech and Thomas, 1977), E_{Na} and E_{Ca} would be at +76mV and +214mV respectively. At potentials more negative than these values the flow of these ions would result in an inward current.

The inward flow of Cl^- ions or outward flow of K^+ ions could both account for unitary outward currents at the potentials observed. With an internal Cl concentration of 12mM (cf. Thomas, 1977; Meech and Thomas, 1977), E_{Cl} would be approximately -53mV. Whilst the value of E_K, assuming an internal K concentration of 98mM, would be around -75mV. A channel which allowed the flow of Na^+ ions in addition to K^+ ions may also result in unitary outward

currents at these potentials. Cation nonselective channels have been reported in other systems (Colquhoun, Neher, Reuter and Stevens, 1981; Maruyama and Petersen, 1982; Yellen, 1982). The cation nonselective channels recorded by these workers had similar selectivity for Na^+ and K^+ ions, giving unitary currents which reversed at around 0mV in normal conditions. However, to explain the outward currents observed in the C1 neurone these channels would have to be significantly more permeable to K than Na. The combined equilibrium potential for these ions is around 0mV in normal conditions. Clear outward currents for both the 54pS and 14pS channels were recorded at 0mV patch potential.

The ability to fit the I-V relationships of both the 14pS and 54pS channels with the relationships predicted by the GHK equation for a K current, with extrapolated reversal potentials at E_K, suggested that the unitary currents resulted from a flow of K^+ ions. This was substantiated by the observation of unitary currents of a reduced amplitude when a high K solution was present in the patch pipette. Experiments on isolated outside-out patches confirmed the ionic nature of the large channels. Small channels, corresponding to the 14pS channels were not observed in outside-out membrane patches. However, the experiments on cell-attached patches indicated that these, small single channel currents, are probably due to the flow of K^+ ions.

Unitary outward currents recorded from cell-attached patches were never observed to reverse, even at potentials more negative than E_K. This could be explained by rectification of the current flow through the channels, making the amplitude of unitary events too small to be resolved above the background noise at negative potentials. Alternatively, it could be due to a voltage-dependency of channel gating which causes the channels to be open at depolarised potentials but closed at hyperpolarised potentials. Some increase in the probability of opening of the 54pS channel on depolarisation was observed in cell-attached patches (see section 4.5).

4.2.1 CHANNELS RECORDED FROM OUTSIDE-OUT MEMBRANE PATCHES

Only the larger unitary outward currents were observed in recordings from isolated outside-out membrane patches. In all these experiments the patch pipette contained a 96mM K solution. The reversal potential of the channels was found to shift in accordance with the Nernst equation when the K concentration of the bathing solution was altered. When isolated outside-out membrane patches were bathed with 5mM K, in order to mimic the physiological K gradient, the unitary outward currents rectified in the same manner as observed in cell-attached patches. This rectification could be fitted by the theoretical relationship calculated from the GHK equation. Under these conditions E_{Cl} was at -7mV. This therefore demonstrates that these unitary currents could not be due to the flow of Cl^- ions. In outside-out patches with the same 96mM

K solution on either side of the membrane, non rectifying unitary currents, which reversed at 0mV, were recorded. Unitary currents in symmetrical 96mM K solutions had a conductance of around 130-170pS.

In four experiments, where the solution bathing the patch of membrane was changed to one containing 44mM K^+ ions, there was a resultant shift in the reversal potential to -19.6 ± 1.0mV. This value closely agreed with that predicted by the Nernst equation (-19.7mV). In these experiments sucrose was used to substitute for KCl to maintain osmotic strength. E_{Na} in this solution was at 0mV. An accurate assessment of the selectivity ratios of the channels for K^+ ions over Na^+ ions was not performed (cf. Yellen, 1984). However, the I-V relationship in asymmetric solution in which E_{Na} was around +88mV, suggested that a significant contribution to the current from Na^+ ions was unlikely.

Unitary currents from outside-out patches bathed in all three K solutions could be fitted reasonably well by the theoretical relationships for a K current calculated from the GHK equation. However, these channels do not obey in all respects the constant field theory assumed by the GHK equation. The constant field theory (Goldman, 1943) predicts that the permeability will remain constant as the concentration of the ion is changed. In order to fit the GHK equation to the unitary currents recorded here, different values for P_K were used in the different solutions. In figure 3.8,A it can be seen that the theoretical lines fitted by the GHK equation crossed over at about +30mV. The constant field

theory predicts that these lines will come to a common asymptote at very positive potentials (see Jack, Noble and Tsien, 1975). Perhaps a significant amount of Na^+ ions were able to flow through the channels at positive potentials, thus altering the apparent fit to the GHK equation.

4.2.2 IONIC NATURE OF CHANNELS OBSERVED ON INSIDE-OUT PATCHES

Experiments on isolated outside-out patches indicated that the large unitary currents recorded were due to current flow through K channels. However, the subsequent finding of two different populations of outward channels in inside-out patches complicated the interpretation that the channels observed in outside-out patches correspond to the 54pS channels in the cell-attached patches.

Both Ca-dependent and Ca-independent channels were found to be present in isolated inside-out patches. These channels could also be classified into two populations on the basis of their slope conductances at +10mV in asymmetrical 5/100mM K solution. The Ca-dependent channels had a mean conductance of 50 ± 11pS (n=6), whilst the Ca-independent channels had a mean conductance of 79 ± 15pS (n=9). Despite the large standard deviations of each set of values, a T-test performed on the data indicated that they belonged to two separate populations ($p<0.01$).

The Ca-dependent channels had a mean conductance value which was closer to that of the large channels observed in cell-attached patches. However, these channels were only observed in 7 out of 33 inside-out patches, while the large unitary outward currents corresponding to the 54pS channel were seen on virtually all cell-attached patches. Ca-independent channels were observed far more frequently on inside-out patches. Despite the uncertainty as to which type of channel observed on inside-out patches corresponds to the 54pS channel of the cell-attached patch or the channels observed on outside-out patches, it is likely that both these types are selective for K^+ ions. Extrapolation of the I-V relationships of both Ca-dependent and Ca-independent channels give reversal potentials more negative than -50mV (figures 3.16 and 3.18). This was in a solution in which E_{Cl} was -7 to -9.6mV and E_{Na} was +82mV. Changing the Ca concentration had no effect on the I-V relationships of either type of channel. It can therefore be concluded that both the Ca-dependent and Ca-independent channels are largely, if not exclusively, selective for K^+ ions.

4.3 Ca-DEPENDENT K CHANNELS

Potassium channels whose activity was dependent on intracellular Ca concentration were present in 7 out of 33 inside-out membrane patches. These channels were different from the previously described Ca-dependent K channels of snail neurones (Lux, Neher and Marty 1981; Ewald, et al, 1985). The slope conductance of these channels was around 50pS at +10mV in solutions

approximating a physiological K gradient. This compared with an ohmic conductance of 19pS for the Ca-dependent K channels described by Lux et al (1981) in cell-attached patches of certain neurones of Helix pomatia, which have a predominant Ca-dependent outward current. The Ca-dependent K channels described by Ewald et al (1985) in isolated inside-out patches of Helix aspersa neurones had a conductance of 40-60pS in symmetrical K solutions. In conditions mimicking the physiological K gradient these channels had a conductance of around 20pS. This is similar to the conductance value obtained by Lux et al (1981), but is distinct from the large conductance (200-300pS) Ca-dependent K channels described in many other preparations (for review, see Latorre and Miller, 1983).

The Ca-dependent K channels described here may be similar to the large conductance Ca-dependent K channels described in other preparations such as chromaffin cells (Marty, 1981), rat myotubes (Pallotta, Magleby and Barrett, 1981), and channels from rabbit T-tubules, incorporated into lipid bilayers (Latorre, Vergara and Hidalgo, 1982). If it is assumed that the permeability value of the channels remains relatively constant with changes in the K concentration on either side of the membrane, the expected single channel conductance in symmetrical K solutions can be calculated. Using a permeability value of 2.29×10^{-13} cm.^{3}s^{-1} (from figure 3.16), the expected single channel conductance in symmetrical 140mM K solution was calculated to be approximately 125pS. The conductance of Ca-dependent K channels of rat myotubes was measured by Barrett, et al (1982) to be 218pS with symmetrical 144mM K solutions on either side of the patch and at a temperature of 21-24°C. The

Ca-dependent K channel of chromaffin cell membranes was shown to have a single channel conductance of 180pS in symmetrical 144mM K solution (Marty, 1981).

There is some evidence that the permeability value of the channels may not remain constant in different K^+ ion gradients (see figure 3.8). In figure 3.8,A, the single channel conductance in a symmetrical 96mM K solution was 130pS. In a physiological K gradient these channels had a slope conductance at +10mV of 50pS. Although this is near to the mean conductance value of Ca-dependent K channels in an inside-out patch, this does not prove that these channels were Ca-dependent. Using the permeability value obtained in the symmetrical 96mM K solution ($P_K=3.56x10^{-13}cm.^3s^{-1}$), a conductance value of 200pS in symmetrical 140mM K solution was calculated. Clearly, these channels have a higher conductance than the previously reported Ca-dependent K channels of snail neurones, and appear to belong to the class of high conductance Ca-dependent K channels.

One common feature of the high conductance Ca-dependent K channels is their ability to maintain a high selectivity for K^+ ions whilst having a large conductance (Latorre and Miller, 1983). Detailed experiments on the selectivity of the Ca-dependent K channels recorded here, for K^+ ions over Na^+ and Cl^- ions, was not carried out. However, the fit by the GHK equation for a K current, with an extrapolated reversal potential to -75mV in an asymmetrical solution in which E_{Na} was +82mV and E_{Cl} was between -7 and -9.6mV, suggested that these channels were highly selective for K^+ ions

(see figure 3.16).

4.3.1 RELATIONSHIP BETWEEN pCa AND PERCENTAGE OPEN TIME

In two experiments it was possible to determine the relationship between the percentage open time of Ca-dependent channels and Ca concentration (expressed as pCa). These relationships were limited as only four different Ca concentrations were used. However, no results were obtained from later experiments in which six different Ca concentrations were available, since Ca-dependent K channels were no longer found (possibly due to a seasonal variation, see below). The percentage open time of Ca-dependent K channels increased with increasing Ca concentration. The relationship between pCa and percentage open time is shown in figure 3.15. This relationship is likely to be sigmoidal as seen by Barrett et al (1982), for Ca-dependent K channels of rat myotubes. However, further points in the pCa range 8 to 7 would be required to determine the true relationship for these channels. Barrett et al (1982) observed a steep increase in percentage open time at a pCa value of 6. In the experiment shown in figure 3.15 a steep increase over the pCa range 8 to 7 seems apparent. However, further points would be required to confirm this.

In calculating the percentage open time it was assumed that the number of channels present in the patch of membrane was equivalent to the maximum number of simultaneously open channels. It was also assumed that all the channels present in the patch of

membrane had the same properties and were independent of one another. When Ca-dependent K channels were present in isolated inside-out membrane patches, they were always present in large numbers of about 4 to 8 channels per patch. This was in contrast with the Ca-independent channels which were generally present in smaller numbers (1 to 3 channels per patch). Since Ca-dependent channels always occurred in large numbers, it was not possible to perform a statistical analysis of open and close time kinetics in different Ca concentrations, as performed by Magleby and Pallotta (1983a,b) on single Ca-dependent K channels of rat myotubes.

4.3.2. A POSSIBLE SEASONAL VARIATION IN THE EXPRESSION OF Ca-DEPENDENT K CHANNELS

The clustering together of Ca-dependent K channels may have been part of the reason why they were found on so few isolated inside-out membrane patches (7 out of 33 patches). However, a seasonal variation in the expression of these channels was also suspected. In the months of February and March, 7 out of 11 inside-out patches from C1 neurones contained Ca-dependent K channels. Whilst, after the middle of April, none of the isolated inside-out patches recorded from contained channels whose activity increased with increasing Ca concentration (17 patches). Experiments performed previously in the summer months showed no Ca-dependency of channels observed in inside-out patches (4 patches).

A change in the activity of the snails was noticed after April. In the winter months the animals were more difficult to arouse from hibernation, and tended to retreat into their shells, even when kept in a moist environment with food. However, after April the animals were far more active once aroused from hibernation. The MCC which is the homologous neurone to the C1 neurone in other gastropod molluscs has been found to be involved in the arousal and modulation of feeding behaviour in Aplysia and Limax (Kupferman and Weiss, 1981; Gelperin, 1981; see section 1.5.3). This neurone is not a command neurone to initiate feeding, but its activity enhances feeding behaviour once started. It is therefore reasonable to suppose that a seasonal variation in the activity of this neurone may occur. An increased Ca-dependent K current might be expected to stabilise the membrane potential towards E_K and shorten the duration of any action potentials elicited. An increase in the Ca-dependent K current of inactive snails has recently been reported (Madden, 1986). Seasonal variations in the 5-HT content of Helix brains has also been reported (Juorio and Killick, 1972).

Although Ca-dependent K channels were not detected in patch clamp experiments after April, voltage-clamp experiments at this time still showed a significant Ca-dependent outward current. This was evident as a slight N shape in the I-V relationship of the C1 neurone at very positive potentials. Bathing the cells in a nominally zero Ca solution greatly reduced this component (figure 3.24). Since a significant Ca-dependent K current was apparent in

voltage clamp experiments, one would expect to be able to detect the channels corresponding to this current. Voltage clamp experiments, looking at the Ca-dependent outward current, were not performed during the winter months, therefore, it is not known if the proportion of outward current which was Ca-dependent changed at all during this time. A variation in the N shaped I-V relationship of a Helix neurone has been reported to depend on the activity of the snails (Madden, 1986). It is possible that other Ca-dependent K channels such as those seen by Lux et al (1981) and Ewald et al (1985) are present in the C1 neurone, but so far have not been detected in isolated patches. Ca-dependent channels may also be restricted to certain areas of the C1 neurone soma, such that they are not readily detected. Although a seasonal variation was suspected, no firm evidence exists for this and a careful and detailed study would be required to determine if this was indeed the case.

4.4 Ca-INDEPENDENT K CHANNELS

Potassium channels whose activity was independent of the internal Ca concentration were observed in 23 out of 33 isolated inside-out membrane patches from the C1 neurone. These channels had a mean slope conductance of 79±15pS at +10mV. There was a large variation in the conductance values and, in general, the I-V relationships of these channels could not be fitted well with the theoretical relationship calculated from the GHK equation for a K current. It was, however, likely that these channels were largely selective for K^+ ions. The extrapolated reversal potentials from

lines fitted by eye to these I-V relationships were more negative than -50mV. Under the conditions of the recording E_{Cl} was between -7 and -10mV and E_{Na} was around +82mV. Varying the Ca concentration had no effect on the I-V relationship. Therefore it can be concluded that there is very little contribution to the current flow from either Cl^-, Na^+ or Ca^{2+} ions.

4.4.1 EFFECTS OF INTERNAL TEA AND Cs

Further evidence that unitary Ca-independent currents were the result of ion flow through K channels was obtained from experiments using TEA and Cs. Both Cs and TEA have been shown to block molluscan K currents. Internal Cs^+ ions have been used to block K currents of molluscan neurones (Akaike, Lee and Brown, 1978; Tillotson and Horn, 1978), while both internal and external TEA blocks K currents of molluscan neurones. Thompson (1977) reported that external TEA (50mM) selectively reduced the voltage-dependent delayed K current, but had little effect on the Ca-dependent K current of the mollusc Tritonia. However, external TEA was found to block both Ca-dependent and voltage-dependent K currents of Helix and Aplysia neurones (Meech and Standen, 1975; Hermann and Gorman, 1981). Hermann and Gorman (1981) also found that internal TEA blocked the voltage-dependent K current, but had less of an effect on the Ca-dependent K current in the Aplysia neurone R15.

Perfusing the inner surface of isolated inside-out patches with TEA (20mM) and Cs (10mM) caused an apparent reduction in the amplitude of Ca-independent K channels. Internal TEA and Cs have been shown to cause an apparent amplitude reduction of Ca-dependent K channels in cultured adrenal chromaffin cells (Yellen, 1984). Internal TEA has also been shown to cause an apparent amplitude reduction of Ca-dependent K channels of smooth muscle cells (Benham, et al, 1985). The amplitude reduction caused by TEA and Cs is thought to be due to a very fast blocking and unblocking of the K channels by TEA^+ and Cs^+ ions (Yellen, 1984). Fast blocking and unblocking causes brief interruptions of single channel currents. If these interruptions are so brief that they cannot be resolved by the recording system, the block is seen as a reduction in the amplitude of the unitary current events.

From the experiments illustrated in figures 3.19 and 3.21, it appears that the block produced by 10mM Cs was slower than that produced by 20mM TEA. A small increase in open channel noise was apparent with Cs, but not with TEA. However, no attempt was made to quantify these observations. It appeared that 10mM Cs was more effective in blocking the Ca-independent channels of the C1 neurone than it was at blocking the Ca-dependent K channels of adrenal chromaffin cells (Yellen, 1984). In the experiment shown in figure 3.21, Cs reduced the channel amplitude to 45% of control levels. Internal TEA reduced the single channel amplitude to 25% of control levels. No attempt was made to determine the voltage-dependency of the blocking action of Cs and TEA as determined by Yellen (1984).

4.4.2 COMPARISON OF Ca-INDEPENDENT CHANNELS WITH CHANNELS RECORDED FROM CELL-ATTACHED PATCHES

Since isolated inside-out patches containing only one Ca-independent K channel were sometimes observed, it was possible to obtain measurements of open and closed times for these channels. In a very limited analysis, the open and closed times of the 54pS K channels in cell-attached patches and the Ca-independent K channels in isolated inside-out patches were compared (see Table 3.1). The Ca-independent channels on isolated patches generally showed very different activity from that of the 54pS channel on cell-attached patches. Ca-independent channels spent a far greater percentage of time in the open state (60 to 75%) as compared to channels in cell attached patches which had percentage open times of <1 to 37%. The longer closed times evident in channels recorded from cell-attached patches, were generally not evident in recordings of single Ca-independent channels from isolated inside-out membrane patches.

The difference in channel kinetics and conductance of these two types of channels suggested that they belonged to different populations and that perhaps the Ca-independent channels were not expressed when recordings were made in the cell-attached mode. However, it is possible that these were the same channels whose characteristics were altered on isolation of the patch of membrane from the cell. It has been reported that single Cl channels of epithelial cells change their kinetic characteristics on isolation

of the membrane patch (Kolb, Brown and Murer, 1985). The majority of isolated inside-out patches contained Ca-independent K channels, whilst, in virtually all cell-attached membrane patches, unitary currents considered to belong to the large, 54pS group of channels were recorded. From this observation, it would seem likely that these are the same channel, but that the properties of the channel have been altered on isolation of the patch of membrane. In order to determine if this is the case, it would be necessary to first record the channels in the cell-attached patch and then isolate the patch to test the Ca-dependency of the channels. In a few records in which Ca-independent channels were recorded on inside-out patches, large outward channels of normal amplitude (presumably corresponding to the 54pS channels) were first observed at a few potentials in the cell-attached patch.

Changes in single channel kinetics upon isolation of the patch of membrane could be explained by the loss of some intracellular regulatory substance. It has been shown that phosphorylation of a site at, or near, K channels can modify their activity (Shuster et al, 1985; Ewald et al, 1985).

4.5 MODULATION OF K CHANNEL ACTIVITY BY 5-HT

Application of 5-HT onto the C1 neurone was found to decrease the activity of the large 54pS K channels recorded in the cell-attached mode. This action of 5-HT, applied from outwith the patch pipette, was indirect and suggested the involvement of an intracellular messenger. A similar effect of 5-HT on K channels

recorded from Aplysia sensory neurones was previously reported by Siegelbaum et al (1982). In the experiments of Siegelbaum et al (1982) the K channels recorded were closed in an all-or-nothing fashion by addition of 5-HT to the bathing medium surrounding the cells. The channels which remained open in the presence of 5-HT maintained the same gating kinetics as in control conditions.

It was difficult to determine if a similar all-or-nothing closure of K channels also occurred in the experiments on the C1 neurone. In most experiments, only two or three channels were active under control conditions and during application of 5-HT the channels remained in the closed state. In a few experiments brief isolated openings of channels were apparent in the presence of 5-HT. These occasional channel openings contrasted with the frequent openings of channels in control conditions and suggested that 5-HT may cause a change in the probability of opening of the channels. However, since the number of channels present in the patch of membrane was always greater than 1, it was difficult to undertake the kinetic analysis required to determine the mechanism by which 5-HT decreased K channel activity in the C1 neurone.

The channels observed in Aplysia sensory neurones had similar conductance values to the K channels modulated by 5-HT in the C1 neurone. However, these were recorded under very different ionic conditions. A more valid comparison is that of the permeability values, which takes into account the concentration of K^+ ions. The permeability of the 5-HT sensitive K channels of Aplysia sensory neurones was somewhat lower than that of the 5-HT sensitive K

channels of the C1 neurone ($8.7x10^{-14}cm.^3s^{-1}$, compared with $2.3x10^{-13}cm.^3s^{-1}$).

The 5-HT sensitive K channels of Aplysia sensory neurones were found to be insensitive to intracellular free Ca levels. These unitary K currents could be fitted by the predicted I-V relationship calculated from the GHK equation for a K current, thereby showing a voltage-dependency in their conductance. However, the probability of channel opening was found to be only slightly dependent on potential with a 2-fold increase for around 52mV depolarisation. These K channels were present at resting potential and were thought to contribute to a background current. Their properties were consistent with the properties of the serotonin sensitive S current, studied at a macroscopic level.

Attempts were made to determine the effect of potential on the percentage open time of the channels in the C1 neurone. A large variability was found between patches. In general, an increase in percentage open time with depolarisation was observed for the 54pS K channels recorded in cell-attached patches. This increase was of the order of 2 fold for a 13.7 ± 7.5mV depolarisation (n=4). Single channel currents in the C1 neurone were rarely seen at resting potential. Moreover the percentage open time of these channels, even at depolarised potentials, was lower than that of the K channels of Aplysia sensory neurones (1 to 40%, compared to 60 to 90%).

The Ca-dependency of the 5-HT sensitive channels of the C1 neurone was uncertain, due to the later finding of both Ca-dependent and Ca-independent channels in isolated inside-out membrane patches from the C1 neurone. In retrospect, this uncertainty could have been solved by firstly testing the effect of 5-HT on channels in the cell-attached mode, before isolating the patch of membrane to determine the Ca-dependency of the channels. Although the frequency with which Ca-independent channels were observed suggested that the 5-HT sensitive channels observed in the cell-attached patch may be Ca-independent, the mean conductance of the Ca-dependent channels was closer to that of the 5-HT sensitive channels observed on cell-attached patches. An effect of 5-HT on Ca-dependent K channels would also explain a previous finding that the 5-HT response in voltage-clamped C1 neurones is blocked by 1mM Co. However, the effect of Co^{2+} ions in blocking the 5-HT response may be an nonspecific effect.

4.6 ACTION OF Co^{2+} IONS IN BLOCKING THE 5-HT RESPONSE

Earlier voltage clamp experiments on the C1 neurone had shown that the 5-HT response was sensitive to K channel blocking agents and to increasing the extracellular K concentration. The response was therefore most probably due to a decrease in gK. However, it was thought that the K current affected may be Ca-dependent since the response to 5-HT was also blocked by 1mM Co, which is known to block the Ca-current of molluscan neurones (Akaike, Brown, Nishi and Tsuda, 1981).

In studies on a similar voltage-dependent 5-HT response in neurones of the viscero-abdominal ganglionic mass of Helix, Deterre et al (1981) reported that this response was sensitive to extracellular Co and changes in extracellular free Ca, but not to intracellular free Ca levels. They concluded that, although the 5-HT induced decrease in gK was sensitive to extracellular Ca, it was probably not a reduction of a Ca-dependent gK, which is sensitive to intracellular free Ca levels.

4.6.1 SINGLE CHANNEL CURRENTS IN THE PRESENCE OF Co

Experiments on K channels were performed either with Co present in both the bath and patch pipette, or in the patch pipette alone. In the presence of Co single channel K currents of normal conductance were observed. However, when Co was present in both the bath and patch pipette application of 5-HT to the C1 neurone was no longer effective in reducing the activity of the 54pS K channels. With Co present in the patch pipette alone, 5-HT was still effective in reducing the activity of the 54pS K channels. These results suggest that the action of Co in blocking this response was not by reducing the K current, but by some other mechanism. Co may act at the level of the 5-HT receptor to prevent the action of 5-HT. Alternatively, Co may interfere with the production or action of the second messenger by its antagonism with Ca. A requirement for Ca in the production of cAMP has been reported (Brostrom, Huang, Breckenridge and Wolff, 1975; Brostrom, Brostrom, Breckenridge and Wolff, 1978). However, Deterre et al

(1981) found that the response of Helix neurones to intracellularly injected cAMP was also blocked by Co.

4.6.2 COMPARISON OF THE EFFECTS OF Co, VERAPAMIL AND LOW Ca SOLUTION ON THE RESPONSE TO 5-HT

Whilst 1mM Co virtually abolished the response to ionophoretically applied 5-HT, the organic Ca current blocking agent, verapamil (at a concentration of 50μM) had no effect on the 5-HT response. This concentration of verapamil has been shown to significantly reduce the Ca current of Helix neurones (Akaike, et al, 1981). However, verapamil may not be a very specific blocker of the Ca current. In experiments performed here on the I-V relationship of the C1 neurone, verapamil greatly reduced the outward current, apparently blocking more than just the Ca-dependent component of this current (see figure 3.29). It has also been reported that verapamil can directly block Ca-dependent K channels as well as acting by reducing gCa (Gola and Ducreux, 1985).

A low Ca solution was found to be more effective than a 1mM Co solution in reducing the Ca-dependent component of the outward current at depolarised potentials (compare figures 3.25 and 3.27). However, perfusing the preparation with a zero Ca solution had a variable effect on the response to ionophoresed 5-HT. When the C1 neurone was held continuously under voltage clamp conditions, the response to 5-HT was slightly increased in the presence of a nominally zero Ca solution. If the response was the result of a

reduction in a Ca-dependent outward current, it would be expected to be decreased in a low Ca solution. This anomalous result may have been due to a change in the surface charge of the membrane since Ca^{2+} ions are known to be important in the maintenance of the surface charge.

When the cell was held in current clamp conditions at resting potential, during perfusion with the nominally zero Ca solution, a reduction in the 5-HT response was observed on returning to voltage clamp. However, this effect of low Ca was irreversible. The effect of low Ca solution on the Ca-dependent outward current was reversible.

The lack of convincing decreases in the 5-HT response with 50 μM verapamil or a nominally zero Ca solution, further suggested an nonspecific effect of Co in blocking the 5-HT response. Although there may be problems with the specificity of the treatments with verapamil and low Ca, both treatments would be expected to significantly reduce Ca entry during depolarisation, thereby reducing a response which was dependent on the background level of Ca-dependent K current. Even in a nominally zero Ca solution, a significant amount of Ca^{2+} ions are present. However, this solution was clearly effective in reducing the Ca-dependent component of the outward current.

4.7 EFFECT OF 5-HT ON THE Ca-DEPENDENT COMPONENT OF THE I-V RELATIONSHIP

An N shaped I-V relationship, revealing a Ca-dependent component of the K current, was observed in the C1 and F1 neurones (cf. Meech and Standen, 1975). The difference between the currents in 10mM Ca and nominally zero Ca solution gave a bell shaped curve, which corresponded to the Ca-dependent K current. In these experiments, the peak of the curve was around +80 to +90mV. In the experiments of Meech and Standen (1975) this peak was around +70mV. Lux and Hofmier (1982) showed that the potential at which this peak occurred depended on the time during the pulse at which the current was measured, moving to depolarised potentials for increasing time. Currents in the experiments reported here were measured after 100ms (80ms in some cases). At 100ms, the peak of the curve shown by Lux and Hofmier occurred around +70mV.

In 5 experiments 50μM 5-HT flattened the N shaped I-V relationship of the C1 neurone. However in a further 7 experiments no effect of 5-HT was observed. The experiments in which a flattening of the N shape was observed indicated that, in these cells 5-HT caused a reduction of the Ca-dependent K current. Experiments in which no effect of 5-HT was observed may have been due to inadequate perfusion of the bath, but tests with methylene blue dye indicated that the perfusion system was efficient in changing the contents of the bath. Another explanation for this lack of effect may be that in some cells 5-HT has little, or no,

effect on the Ca-dependent component of the outward current. Previous experiments, showing a flattening of the I-V relationship by 5-HT, did not look at such depolarised potentials (Cottrell, 1982b).

4.8 THE EFFECTS OF INTRACELLULAR EGTA AND Ca INJECTION

Intracellular injection of the Ca chelator, EGTA, was used in an attempt to buffer Ca entering the cell during depolarisation. In this way, prolonged EGTA injection has been used to decrease the Ca-dependent outward current (Deterre et al, 1981: Kehoe, 1985b). 15 minute injections of EGTA had little effect on the I-V relationship of the C1 neurone, measured up to -10mV. This suggested that longer injections of EGTA may have been required in order to load the cell with EGTA and block the Ca-dependent K current. However, intracellular EGTA did appear to have an effect on the 5-HT response. The response was increased or unaffected at potentials more negative than -20mV, but reduced at more depolarised potentials. This voltage-dependent effect of EGTA is difficult to explain, but may reflect differing contributions of Ca-dependent and other K currents at different potentials.

If the response to 5-HT is due to a reduction of a Ca-dependent K current, this response should be increased when the Ca-dependent K current is increased by raising the intracellular free Ca levels. Injecting Ca^{2+} ions into molluscan neurones has been shown to increase the Ca-dependent K current, (Meech, 1974; Hermann and Gorman, 1981a,b). Intracellular injection of Ca into

the C1 neurone of Helix aspersa has been shown to activate an outward current whose reversal potential depends on the extracellular K concentration (Cottrell, 1982c).

In the experiments performed here, injection of Ca^{2+} ions induced an outward current. However, this outward current declined rapidly with time after the injection. The rapid decline in the outward current was probably due to the highly effective intracellular Ca sequestration mechanisms. It has been suggested (Barish and Thompson, 1983) that the distribution of injected Ca is limited by metabolic uptake processes, such as those in mitochondria. Ca entering due to depolarisation results in an increased Ca concentration around the periphery. It has also been suggested that the Ca buffering capacity is greater at the periphery (Tillotson and Gorman, 1980).

Despite small increases in the outward current of the C1 neurone after Ca injection, no increase in the amplitude of the 5-HT response was observed. However, this experiment was technically difficult due to the rapid decline of the outward current (Davies, 1986). In studies of a similar voltage-dependent decrease in gK induced by the peptide, YGGFMRFamide (Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH2), many attempts were required to obtain a sufficiently increased outward current, which resulted in an increased response to the peptide (Cottrell, 1982c; Cottrell, Davies and Green, 1984; Davies, 1986).

The lack of effect of Ca injection on the 5-HT response observed here may have been due to an uneven distribution of Ca at the membrane. Ca injected from the tip of an electrode would not diffuse evenly throughout the cell, so that only a portion of the membrane might experience an increase in Ca concentration (Barish and Thompson, 1983). Furthermore, the diffusion of Ca within the cell is restricted by the sequestration mechanisms (Rose and Lowenstein, 1975). Therefore it is possible that the K current activated by an increase in intracellular Ca, is on a portion of the membrane which does not receive application of 5-HT.

4.9 CONCLUSIONS ON THE Ca-DEPENDENCY OF THE 5-HT RESPONSE

The results concerning the Ca-dependency of the 5-HT response are rather confusing. Some experiments, such as those with EGTA injection and the effect of 5-HT on the N shape of the I-V curve, suggest that there may be some Ca-dependency. However, the experiments with verapamil and Ca injection point towards a lack of Ca-dependency in the response. The Ca-dependent K channels, although having a conductance closer to that of the 5-HT sensitive K channels recorded from a cell-attached patch, were only infrequently observed. Channels sensitive to 5-HT were found in most patches. This 5-HT response may therefore be due to a decrease in gK which is not Ca-dependent. However, it is also possible that the activity of more than one type of K channel is suppressed by 5-HT. The contributions of the different types of K current may vary between C1 neurones, and therefore, the extent of

their block by 5-HT may vary. The different effects of 5-HT on the N shaped I-V relationship suggests that this may be the case.

In view of the confusing results for the Ca-dependency, and the existence of both Ca-dependent and Ca-independent K channels, it is possible that both types of channel may be affected by 5-HT. Experiments in which the Ca-dependency of the channels is examined after their sensitivity to 5-HT has been established, would be required to solve this problem. Increases in intracellular cAMP have been found to affect more than one type of K current in bag cell neurones of Aplysia (Strong and Kaczmarek, 1986).

4.10 COMPARISON OF THE 5-HT RESPONSE OF THE C1 NEURONE WITH OTHER SIMILAR RESPONSES

The 5-HT induced inward current observed in certain Aplysia neurones by Pellmar and co-workers had similar voltage dependent properties to the 5-HT response of the C1 neurone (Pellmar and Carpenter, 1979;1980; Pellmar, 1984). However, unlike the response of the C1 neurone, this response was found to be insensitive to changes in the extracellular K concentration and was shown to be the result of an increase in gCa. A 5-HT induced increase in gCa has recently been found in some neurones of Helix (Paupardin-Tritsch, Hammond and Gerschenfeld, 1986; Paupardin-Tritsch, Hammond, Gerschenfeld, Nairn and Greengard, 1986). However, previous experiments on the C1 neurone have shown no effect of 5-HT on the Ca current (Barnes, Cottrell and Dunbar, 1984; Cottrell, unpublished observations).

The 5-HT response of the C1 neurone also bears many similarities to the response of Aplysia sensory neurones to 5-HT (Klein and Kandel, 1978; 1980; 1982; Siegelbaum et al, 1982). Both of these responses have been found to be due to a decrease in gK. However, some differences are also apparent. The response of the C1 neurone has been shown to be blocked by Ba (Barnes, Cottrell and Dunbar, in preparation), but the 5-HT sensitive K current of Aplysia sensory neurones is not sensitive to Ba (Klein, Camardo and Kandel, 1982). Although this response was sensitive to extracellular Co, the authors concluded that it was not due to a decrease in a Ca dependent gK since it was not sensitive to Ba or enhanced by intracellular Ca injection. The Ca dependency of the C1 neurone response to 5-HT remains uncertain and results indicate that a Ca dependent component may be present.

The 5-HT response of some Helix neurones studied by Deterre et al (1981; 1982) and Paupardin-Tritsch et al (1981) also has many similarities to the 5-HT response of the C1 neurone. This response was sensitive to extracellular Ba, as well as Co and extracellular Ca levels. However, this response differed in one important respect from the response of the C1 neurone, since it was observed to reverse at around -50mV. A reversal of the 5-HT induced inward current in the C1 neurone was never observed. In addition no sensitivity of the response to EGTA injection was found by Deterre et al (1981).

4.11 SECOND MESSENGER MEDIATION OF THE 5-HT RESPONSE

The action of 5-HT on single K channels, when applied to the C1 neurone from outwith the patch pipette, strongly suggested that the effect of 5-HT was mediated by an intracellular messenger. Intracellular injection of cAMP causes closure of K channels in Aplysia sensory neurones as does application of cAMP-dependent protein kinase to the inner surface of the membrane (Siegelbaum et al, 1982; Shuster et al, 1985). Some results were obtained here which were consistent with the mediation of the 5-HT response of the C1 neurone by cAMP.

In most experiments the phosphodiesterase inhibitors, IBMX and theophylline, reduced the size of the 5-HT response, but at the same time induced an inward current themselves. This suggested the presence of a basal level of cyclic nucleotides within the C1 neurone, which, when increased, caused a suppression of the same current as suppressed by 5-HT. The inward current induced by IBMX and theophylline was also voltage dependent, increasing in amplitude as the cell was held at more depolarised potentials. However, the ability of raised levels of cyclic nucleotides to mimic the 5-HT response does not provide proof that these substances are physiological mediators of the response.

Small concentrations of phosphodiesterase inhibitors might be expected to potentiate a response which is mediated by cyclic nucleotides. However, a potentiation of the 5-HT response by phosphodiesterase inhibitors was never observed. In one experiment the response to 5-HT was prolonged in the presence of IBMX. Very little effect of IBMX on the I-V relationship of this cell was apparent, suggesting that the basal levels of cyclic nucleotides may have been low. The results of this experiment were consistent with a mediation of the 5-HT response by a cyclic nucleotide.

Injection of cAMP into a C1 neurone, voltage clamped to depolarised potentials, produced an inward current. However the inward current response produced to a 5s injection of cAMP was often prolonged and maintained, thereby preventing further responses. Even responses which were not prolonged were reduced on repeated injections. Such desensitising behaviour was never observed for the 5-HT response.

Intracellular injection of cAMP has been found to mimic the 5-HT response in other cells (Pellmar, 1981a; Deterre *et al*, 1981;1982). The cAMP induced inward current in the C1 neurone is likely to be the result of a decrease in a K current (as is the 5-HT response), since it was only observed when the cell was held at depolarised potentials. However, the ionic nature of the cAMP response was not confirmed.

Care must be taken in concluding that an intracellular messenger which mimics a response, actually mediates the response. Pellmar (1981b) showed that, although the 5-HT response of some Aplysia neurones could be mimicked by intracellular cAMP or phosphodiesterase inhibitors, it was not affected by substances expected to inhibit the production of cAMP. In other instances some 5-HT responses have been shown to be mediated by cAMP. In some Helix neurones 5-HT causes an increase in cAMP production (Deterre et al, 1982). It had previously been shown that intracellular injection of cAMP could mimic the 5-HT response of these cells (Deterre et al, 1981). The adenylate cyclase activator, forskolin, also mimicked the response, and low concentrations of phosphodiesterase inhibitors were capable of enhancing the 5-HT response, (Deterre, 1982). Mediation of the 5-HT response of Aplysia sensory neurones by cAMP has also been established (Castelluci et al, 1982). Injection of the specific inhibitor of cAMP dependent protein kinase, protein kinase inhibitor, blocked presynaptic facilitation and the response to 5-HT in Aplysia sensory neurones.

The demonstration that 5-HT actually causes an increase in cAMP levels in the C1 neurone, and that specific inhibitors such as protein kinase inhibitor block the effect of 5-HT, would be required to establish cAMP as the physiological intracellular mediator of the 5-HT response. The effects of cAMP are thought to be mediated by cAMP-dependent protein kinase, which in turn is responsible for phosphorylating certain proteins. Changes in ionic

conductances mediated by cAMP may be due to phosphorylation at or near the ion channel (Shuster et al, 1985; Ewald et al, 1985). However, other intracellular messengers also operate through protein kinases such as, cGMP-dependent protein kinase, protein kinase C and Ca-calmodulin-dependent protein kinase. It is possible that more than one protein kinase may be capable of phosphorylating the same site, therefore making it essential to determine which one is involved in actually mediating a response. An involvement of a Ca-dependent protein kinase such as protein kinase C may explain the results which suggest a role of Ca^{2+} ions in the response.

4.12 SINGLE CHANNEL CURRENTS OBSERVED ON THE A AND M NEURONES

The single channel currents observed most frequently on the A neurone were different to those seen on the C1 neurone since they were found to reverse between -40 and -50mV. This reversal potential is closer to E_{Cl} than E_K and therefore these channels may be Cl channels. Unitary currents reversing around this potential could also be due to channels which were largely selective to K^+ ions, but allowed a certain flow of Na^+ ions as well. Experiments to determine the ionic nature of the unitary currents recorded from the A neurone were not performed.

4.12.1 EFFECT OF 5-HT ON THE SINGLE CHANNEL CURRENTS OF THE A NEURONE

The channels described above in the A neurone were found to be activated when 5-HT was applied to the cell by microperfusion. This finding was not expected from previous results with voltage clamp experiments, in which 5-HT was shown to elicit a voltage dependent inward current in the A neurone (Cottrell, 1981). The observed activation of outward going channels at depolarised potentials would result in an outward current in the A neurone.

The activation of these channels by 5-HT may have been an artefact due to the method of application of 5-HT. 5-HT was applied by microperfusion. However, this resulted in a dispersal of 5-HT over a large area of the ganglia. On some occasions application of 5-HT was observed to cause a contraction of the salivary ducts and buccal musculature attached to the ganglia. This may have caused movement of the cell, therefore stretching the patch of membrane. The activation of channels by stretch has been reported in cultured chick embryonic skeletal muscle cells. These channels were cation selective, but allowed the passage of both Na^+ and K^+ ions. Ca-dependent K channels and nicotinic channels in the same preparation were not affected by stretch (Guhary and Sachs, 1984). With high K solution in the patch pipette, single channel currents of the C1 neurone were observed to be activated by applying negative pressure to the pipette to obtain a gigaohm seal. In later experiments with the A neurone, attempts were made

to remove the salivary ducts and as much buccal musculature as possible. Activation of these channels by 5-HT was still observed. However, since the buccal ganglia are attached to the buccal mass it was difficult to remove all of the buccal musculature.

Synaptically released 5-HT causes membrane oscillations in the A neurone at resting potential (Cottrell, 1971;1981). 5-HT has been shown to cause oscillatory responses in Xenopus oocytes in which 5-HT receptors were induced by the injection of messenger RNA (Gundersen, Miledi and Parker, 1983). The oscillatory responses of Xenopus oocytes was found to be the result of a Ca-dependent Cl current (Parker, Gundersen and Miledi, 1985). It was suggested that this oscillatory response most resembled the oscillatory responses to 5-HT in the A and M neurones of Helix (Gundersen et al, 1983).

4.12.2 SINGLE CHANNEL CURRENTS OF THE M NEURONE

Only one I-V relationship of single channel currents was obtained from the M neurone. Therefore, it cannot be said if the channels observed were typical. These channels were not observed to reverse, but showed rectification which could be fitted by the theoretical relationship for a K current, calculated from the GHK equation. However, the P_K value calculated from the data points was 1.24×10^{-13} cm.^{3}s^{-1}, which is between the value of the 2 sizes of channel commonly observed on cell-attached patches of the C1 neurone.

Application of 5-HT to the M neurone, whilst recording from a cell-attached patch, resulted in action potentials (which could be recorded extracellularly with the patch pipette) and an increase in channel amplitude, both probably as a result of a depolarisation of the M neurone. This experiment therefore illustrates that, recording from a cell-attached patch during application of a substance is not a suitable technique if any change in the resting membrane potential occurs. In this case, an intracellular voltage clamp would also be required to overcome this difficulty. This problem with the M neurone may be because it responds to 5-HT with a Na-dependent depolarisation in addition to a voltage-dependent depolarisation. However, the Na-dependent depolarising response of the M neurone is rapidly desensitising (Cottrell and Macon, 1974), whilst the depolarisation observed during recording from the cell-attached patch lasted over several minutes. Synaptically released 5-HT, in addition to eliciting the Na-dependent depolarisation in the M neurone, also causes a more persistent depolarising response, which is often seen as a series of membrane oscillations in a hyperpolarised M neurone (Cottrell, 1982b). This more persistent response may have been responsible for the depolarisation observed when 5-HT was applied during patch clamp recording.

In the C1 neurone no response to 5-HT was normally observed at resting potential. Therefore, the effect of 5-HT on channels recorded in the cell-attached patch could be examined, without a change in the cell membrane potential occurring. These limitations

of the cell-attached configuration of the patch clamp technique have been discussed by Fischmeister, Ayer and DeHaan (1986). Simultaneously voltage clamping the C1 neurone, whilst recording from a cell-attached patch, would however, have been useful to ensure that no change in the resting potential of the cell occurred. A hyperpolarising shift in the membrane potential may have been responsible for the reduced channel amplitude seen in some records on recovery from the 5-HT effect.

4.13 PHYSIOLOGICAL ROLE OF THE VOLTAGE-DEPENDENT 5-HT RESPONSE

The C1 neurone of Helix contains 5-HT and has been shown to act on certain follower neurones in the buccal ganglia by the release of 5-HT (see Cottrell, 1977). The unusual response of the A neurone to C1 neurone stimulation was first shown by Cottrell in 1971. This was later established to be a voltage-dependent inward current which was mimicked by application of 5-HT, when recorded under voltage clamp conditions (Cottrell, 1981). The physiological role of this response was studied by Cottrell (1982a). The number of spikes elicited by repeated square wave depolarising spikes in the A neurone decreases, due to accommodation. Stimulation of the C1 neurone was found to overcome the accommodation of the A neurone. A prolongation of A neurone action potential duration was also observed during C1 neurone activity.

Synaptic connections between the two symmetrically positioned C1 neurones have recently been found (Cottrell, unpublished observations). When one C1 neurone is held under voltage clamp conditions at a depolarised potential, stimulation of the contralateral C1 neurone produces a small inward current. Since the C1 neurones are known to contain and release 5-HT, this inward current was most probably due to synaptically released 5-HT. It was reduced at holding potentials more negative than -20mV, suggesting that it showed a similar voltage-dependency to the 5-HT response. The small size of the synaptically evoked response suggested that it occurred on processes some distance from the soma.

The physiological role of the synaptic connections between the C1 neurones may be to overcome accommodation, as shown in the A neurone (Cottrell, 1982a). The increased input resistance resulting would also cause a postsynaptic enhancement of other synaptic inputs onto the C1 neurone. Another possibility is the existence of 5-HT receptors on the synaptic terminals of the C1 neurone. If activation of these receptors resulted in a decrease in gK, as seen in the soma, this may cause a prolongation of action potential duration, and thereby, an increased Ca influx. The increased Ca influx, in turn would enhance transmitter release, giving a positive feedback effect. However, it is not possible to determine, from studies on the C1 neurone soma, if such a response exists at the presynaptic terminals. Some workers have used studies on neurone soma in the assumption that the effect at the

terminals is similar (Klein and Kandel, 1978; 1980). Recently it has been possible to make recordings from crayfish axons close to the synaptic terminals. Here, presynaptic facilitation by 5-HT has been shown to occur by a different mechanism from that occurring in Aplysia sensory neurone soma, and requires the entry of Na^+ ions into the terminal region. The influx of Na is thought to cause an increase in the availability of intracellular Ca (Dixon and Atwood, 1985). It seams more likely that the effect of 5-HT in the C1 neurone is to enhance other synaptic inputs by causing an increase in the input resistance of the cell. Future experiments could be undertaken to determine if stimulation of the contralateral C1 neurone overcomes accommodation, and enhances synaptic inputs to the C1 neurone.

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